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STRUCTURAL AND CHEMICAL ASPECTS OF THE PODOCYST CUTICLE OF THE SCYPHOZOAN MEDUSA, *CHRYSAORA QUINQUECIRRHA*

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The ability of certain organisms to encyst has long provided exceptional opportunities to study the ways in which cells respond to adverse changes in their environment. Among different groups, the structure and chemical nature of cyst walls vary greatly and, knowledge regarding their morphological and chemical characteristics is of particular importance with regard to our understanding of the physiological and biochemical processes involved during encystment. As comparatively little is known regarding encystment in marine or estuarine organisms, this study was directed to an investigation of the structural and chemical aspects of the podocyst cuticle of the Chesapeake Bay sea nettle, *Chrysaora quinquecirrha.*

Most Scyphozoans, at some stage in their life cycle, exist as either a free-swimming medusa or as a small, relatively sessile polyp. The polyps remain attached, via the pedal disc, to hard substrata such as rock or shell. In some species, small bits of tissue containing epidermal and mesenchymal cells separate from the pedal disc and become covered by a thin cuticle. These structures are termed podocysts. It is common for a single polyp to form numerous cysts as it slowly moves along the substrate.

Early work suggests that the formation of podocysts enables the organism to withstand, for prolonged periods, conditions which are unfavorable to the polyp (Tcheou-Tai-Chuin, 1930). In an extensive histochemical survey, Chapman (1968) reports that the podocyst cuticle of *Aurelia* is chitinous and tanned by phenolic substances. The occurrence of chitin in the Cnidaria are contained in numerous review articles (Richards, 1951; Rudall, 1955; Forester and Webber, 1960; Kent, 1964; Jeuniaux, 1971). Hydrozoan chitin has been studied by both chemical and physical means. Rajulu and Gowri (1967), using the coenosteum of *Millipora*, report that the chitin isolated contained, in addition to glucosamine, significant amounts of galactose and mannose. Whether these sugars were actually incorporated in the chitin molecule or arose from associated carbohydrate could not be determined. Rudall (1955) observed that the x-ray diffraction patterns obtained from the Hydrozoan chitin were atypical and stated that this chitin may

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be significantly different from that found in Arthropods with respect to its orientation and crystallinity. Chitin has also been identified via x-ray diffraction studies from the Anthozoan, *Pocillophora* (Wainwright, 1962).

Since the demonstration of chitin in the podocysts of *Aurelia* is based solely on a positive chitosan test (Chapman, 1968) and since the possibility exists that some cnidarian "chitins" may contain sugars other than n-acetylglucosamine, an analysis of *Chrysaora* podocyst chitin was performed using highly specific enzymatic and chromatographic techniques.

MATERIALS AND METHODS

Podocysts from the Chesapeake Bay sea nettle, *Chrysaora quinquecirrha*, were obtained by suspending trays of cleaned oyster shells from a pier located on St. John's Creek near Solomons, Maryland, during late summer and fall. These shells provided a natural substrate on which sea nettle larvae could settle and develop into polyps with subsequent podocyst production. Cyst-containing shells could be maintained for long periods of time, at 10° C, in aquaria containing synthetic sea water (Instant Ocean) maintained at 15 p.p.t. Large numbers of podocysts could easily be obtained by removing them with a fine dissecting needle.

Electron microscopy

Cysts collected as described above were prepared for electron microscopy by fixing them for 2 hours, at 4° C, in a 2.5% solution of glutaraldehyde in 0.005 M Sorenson's phosphate buffer, pH 7.4. The cysts were then post-fixed for 1 hour, at 4° C, in a 1% solution of osmium tetroxide made up in the same buffer, dehydrated through a graded series of alcohols, and infiltrated with a 1 : 1 mixture of Epon and propylene oxide overnight. Cysts were then placed in a 3 : 1 mixture of Epon and propylene oxide for 12 hours and finally embedded in Epon according to the procedure of Luft (1961). Ultra-thin sections were cut with a DuPont diamond knife, collected on collodion-coated, copper grids (200 mesh), and stained with saturated aqueous uranyl acetate (Watson, 1958).

Isolation of chitin

Podocysts, 3.0 mg wet wt, were placed in 10% disodiumethylenediaminetetraacetate (EDTA), pH 8.0, for 12 hours to remove any calcium which may have been present in the cuticle. The podocysts were next washed in distilled water and then sequentially extracted at room temperature in 8.0 M urea (24 hours), 0.01 N NaOH (6 hours) and 1.0 N NaOH at 100° C for 6 hours. The insoluble material (chitin) was washed in distilled water until all traces of NaOH were removed. This material was examined under the light microscope to insure that all podocyst contents were removed and was found to contain clear, transparent podocyst cuticles. The cuticles were collected by centrifugation and hydrolyzed in 6 N HCL in sealed vessels at 100° C for 6 hours. The hydrolysate was then evaporated *in vacuuo* over NaOH, dissolved in distilled water and the procedure repeated. The residue was then taken up in 80% ethanol for chromatography.

Chitin determination

The above material (at least 50 μg) was applied in two adjacent positions on Whatman No. 1 paper and run together with known standards via descending paper chromatography. Standards, 1.0 mg/ml, consisted of glucose, galactose, glucosamine, galactosamine, n-acetylglucosamine and n-acetylgalactosamine. Three solvent systems were used: Isopropanol: water (4:1), Propanol: Ethyl Acetate: water (7:1:1), and n-Butanol: Ethanol: Acetic Acid: water (5:4:3:2). Duplicate runs were made with each solvent system, one being stained with alkaline silver nitrate (Trevelyan, Proctor and Harrison, 1950) for the detection of reducing sugars and the other with Elson-Morgan (Hexosamine Reagent) for the detection of amino sugars (Elson and Morgan, 1933). Mobilities were measured with respect to glucose for all chromatograms stained with silver nitrate (Rg values) and with respect to the solvent front for chromatograms stained with Elson-Morgan reagent (Rf values).

Podocyst cuticle was also tested for the presence of chitin by subjecting cuticle, obtained before and after treatment in sodium hydroxide, to a partially purified preparation of chitinase (from *Streptomyces griseus*) obtained from the Nutritional Bio-Chemicals Corp. The n-acetylglucosamine released was determined by the methods of Reissig, Strominger and Leloir (1955). Standard curves were prepared from known n-acetylglucosamine samples at concentrations from 1–10 μg/ml.

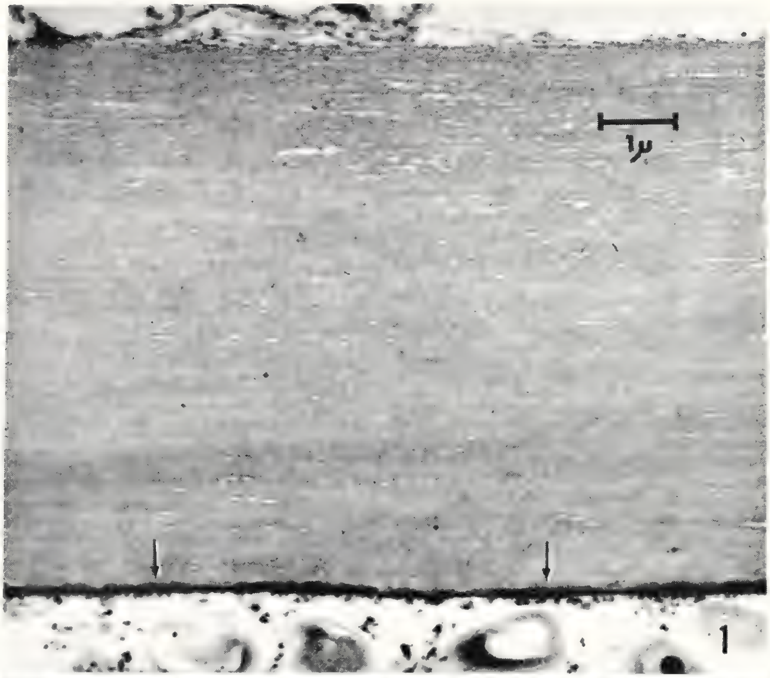
Tyrosinase determination

Cyst samples were prepared by removing several hundred cysts and homogenizing them in 1.0 ml of distilled water at 2° C. Particulate matter was removed via centrifugation at 2° C at 5000 r.p.m. for 15 minutes. The clear supernatant was analyzed for protein via the methods of Lowry, Rosenbrough, Farr and Randall (1951) and tested for tyrosinase activity by a modification of the fluorometric method of Adachi and Halprin (1967). Equal amounts of cyst homogenate and a solution of 0.75 mM L-tyrosine, 100 μM L-Dopa, 0.75 mM ascorbic acid in 40 mM phosphate buffer, pH 6.9 were mixed and allowed to incubate at 25° C. At periodic intervals, 0.2 ml of this reaction mixture was added to 1.8 ml of 10 mM phosphate buffer, pH 6.5, containing 0.0025% zinc sulfate. After mixing, 0.4 ml of 0.25% potassium ferricyanide was added. Exactly two minutes later, 0.2 ml of 5.0 N NaOH, containing 2% ascorbic acid, was used to stop the reaction. Samples were read after five minutes on an Aminco Fluoro-Microphotometer using Corning filter No. 5860 as the primary filter and Corning Nos. 3385 and 4305 as secondary filters. Blanks were prepared using distilled water in place of the cyst homogenate. Activity was determined from a curve prepared using standard tyrosinase samples. Tyrosinase (from mushroom) was obtained from the Worthington Biochemical Corp., Freehold, New Jersey.

RESULTS

Structural characteristics

Chrysaora podocysts are low, dome-shaped structures measuring about 0.3–0.5 mm in diameter. They consist of a mass of whitish tissue covered by a thin,



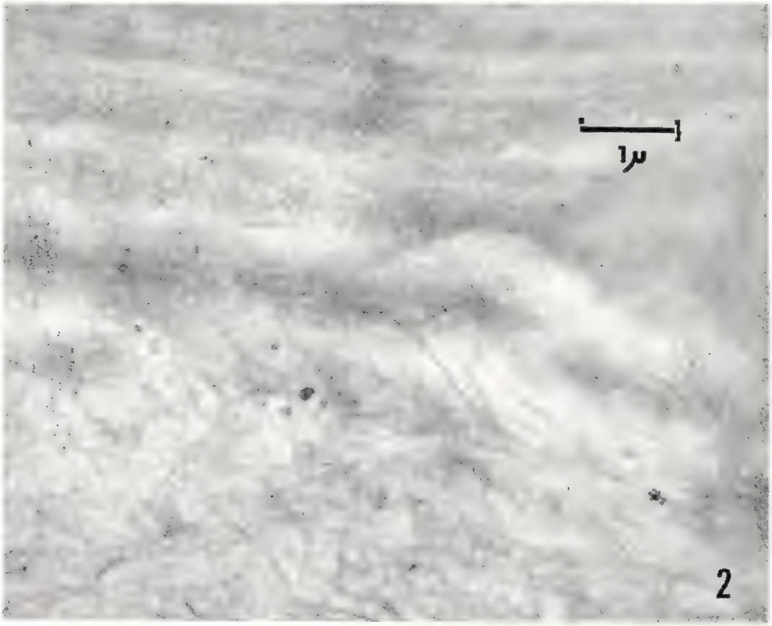


FIGURE 1. A cross section through the cyst wall showing its lamellar structure. Note the darker layer along the surface of cell contact (arrows).

FIGURE 2. An oblique section thru the cyst wall demonstrating the fibrous nature of the lamellae. More compacted lamellae appear at the top of the micrograph.

brown cuticle which varies in thickness from about 9–13 microns. Due to the transparency of the cuticle, large structures, such as nematocysts, can be clearly seen within the intact cyst.

Electron micrographs show this cuticle to be composed of a series of concentric lamellae (Fig. 1). These lamellae appear to be composed of numerous microfibers arranged in sheets (Fig. 2). No structural differentiation can be seen within the cuticle wall except for a narrow layer of varying thickness at the point of contact with the cell mass (Fig. 1, arrow). The cuticular material in this region is heavily stained, appears somewhat granular, and reveals no layered arrangement (Fig. 3).

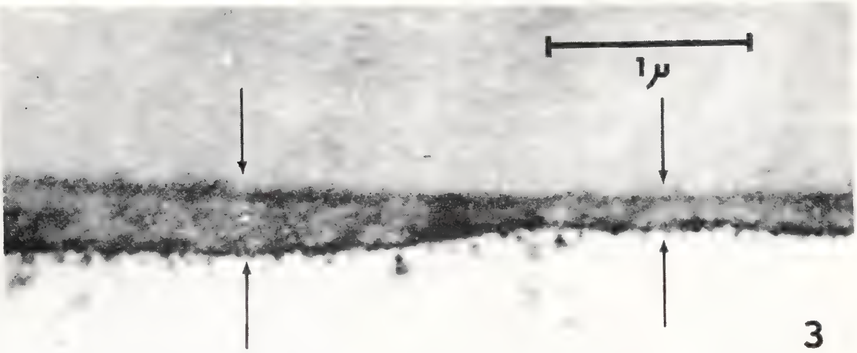
Figure 4 represents a situation in which an outer, thick section of cuticle is separated from a thinner, highly compact section of cuticle by an acellular area of disorganized cuticular lamellae and microfibers. The lamellae at the base of the outer section have separated to a large extent while the lamellae at the periphery are still compact. The inner section of cuticle is highly compact with no trace of lamellar separation. A possible interpretation of these results will be discussed later.

Chemical characteristics

Paper chromatographic analysis of the sugar present in the podocyst cuticle which remained undigested after hot alkali treatment revealed the presence of a single sugar. Comparison of this sugar against known standards in three different solvent systems after development with alkaline silver nitrate or Elson-Morgan reagent showed it to be glucosamine.

Incubation of podocyst cuticle prior to and after hot alkali treatment in the presence of chitinase resulted in the release of N-acetylglucosamine only from cuticles pretreated in hot alkali (Fig. 5). Chitin rarely, if ever, occurs alone in naturally occurring substances but rather as a chitin-protein complex. The necessity for pretreatment of the podocyst cuticle in hot alkali before chitinase degradation could take place indicates that the chitin present in unaltered cuticle is "masked," presumably by its association with protein. This is supported by the tentative identification of several amino acids, *via* paper chromatography, present in the alkali and acid hydrolysates of podocyst cuticles. Due to the extensive degradation of various amino acids during alkali hydrolysis and during acid hydrolysis in the presence of large amounts of sugar, this line of investigation was not pursued.

The possibility that the brownish cuticle of podocysts is the result of a tanning process similar to that which occurs in insect systems was tested by a determination of the polyphenol oxidase (tyrosinase) activity in homogenized cyst extracts. Fluorometric analysis showed an average activity of 0.55 Units/mg protein (Fig. 6). Tanning and hardening of insect cuticle have been shown to be the result of several ortho-dihydroxyphenols present in the cuticle. These phenols are formed by the oxidation of tyrosine by polyphenol oxidase. The resulting polyquinones then crosslink with cuticular protein resulting in a hardening of the cuticle (Locke, 1964; Hackman, 1964).



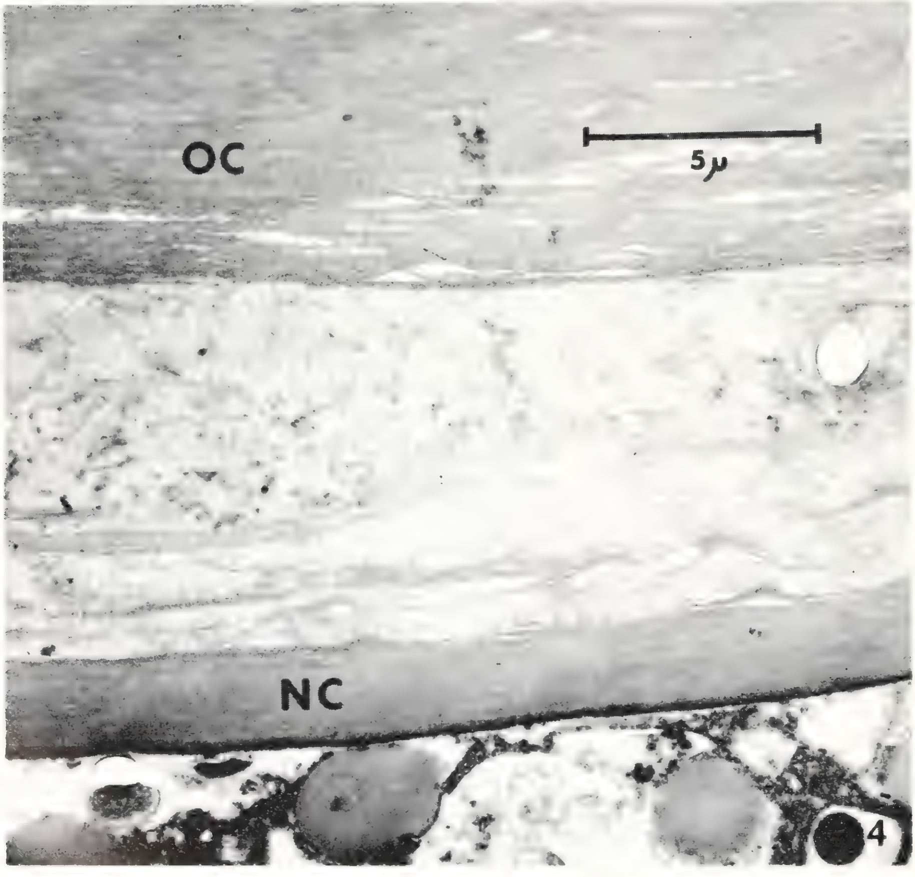


FIGURE 3. A cross section of a cyst wall which has separated from the cellular layer. The darker layer (between the arrows) represents the area of original cell contact. The material composing this layer appears granular and is not arranged into lamella as is the more peripheral material.

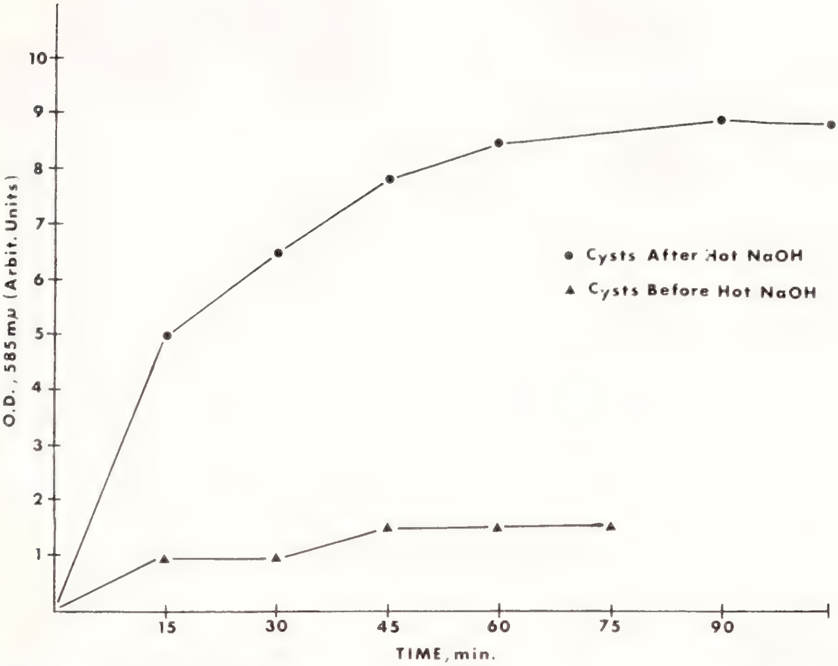


FIGURE 5. The rate of liberation of n-acetylglucosamine from podocyst cuticle in the presence of chitinase prior to (▲) and after (●) cuticle treatment in hot alkali. Spectrophotometric determinations were made at 585 mμ.

DISCUSSION

Enzymatic and chromatographic analysis of the polysaccharide isolated from the podocyst cuticle of *Chrysaora* has provided direct evidence that this material is chitin. No sugars, other than n-acetylglucosamine, were detected. Chemically, chitin is a polysaccharide composed of repeating units of n-acetylglucosamine. Hydrolysis occurs only under extreme conditions (*e.g.*, strong mineral acids) and when chitin is complexed with protein, the resulting material is resistant to enzymatic degradation by chitinase and trypsin (Pryor, 1940). Thus, a chitinous cuticle would provide excellent protection against chemical attack. The demonstration that the chitin in *Chrysaora* cuticle is "masked," presumably due to its association with protein, may explain the negative results obtained by Chapman (1968) with chitinase and with various histochemical analyses in an attempt to corroborate the positive chitosan reaction using *Aurelia* podocysts. In the same study, Chapman demonstrated histochemically the presence of polyphenols in the cuticle of *Aurelia* but did not find polyphenol oxidase activity. The extremely sensitive fluorometric analysis employed in this study demonstrated significant activity in *Chrysaora* cysts and provides further evidence that the cuticle is tanned by a process similar to that found in Arthropods (Hackman, 1964).

FIGURE 4. A cross section depicting an old cuticle (OC) separated from a thinner, compact, new cuticle (NC) by an area composed of disrupted lamellae and microfibrils. Note the darker layer at the point of cell contact in the new cuticle and its absence in the old cuticle.

Structurally, cuticular material is arranged as a series of lamellae which, in turn, are composed of microfibers. Such a situation exists in *Aurelia* cuticle (Chapman, 1968) and is similar to the structure of insect exoskeleton (Locke, 1964). It was not possible, however, to distinguish areas within the podocyst cuticle which correspond to the epi-, exo- and endocuticular regions demonstrated in insect and crustacean exoskeletons. Also, there is a lack of pore canals characteristic of these systems.

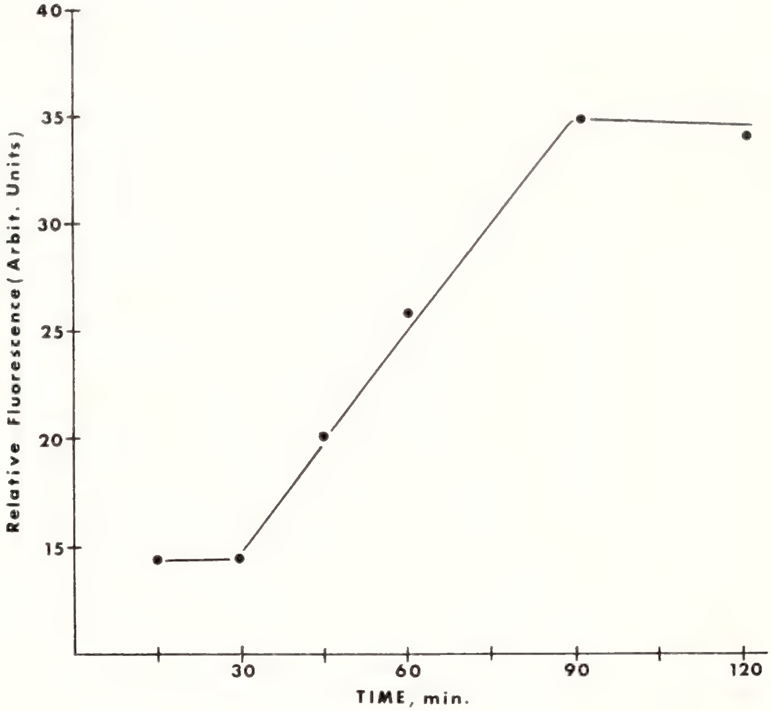


FIGURE 6. Polyphenol oxidase activity of cyst homogenates. The graph represents the rate of conversion of L-tyrosine to L-Dopa in the presence of cyst extracts as measured fluorometrically.

Of particular interest, however, is the fact that, in both insect and cnidarian cuticles, the point of contact with the underlying cells shows different structural characteristics from the immediately overlying more peripheral areas. Schmidt (1956), referred to this area as the subcuticular layer and postulated that it held the insect cuticles to the cells. Locke (1964), however, feels that it is an area of newly secreted endocuticle in which the microfibers are not ordered and have different chemical properties. If such an interpretation could be applied to podocyst cuticle, it would mean that podocyst cells have the continued ability to elaborate new cuticle during encystment. This interpretation is supported by two observations. The first of these regards the situation depicted in Figure 4. In light of what has been shown in Arthropod systems, it is tempting to regard this micrograph as depicting the shedding of an old cuticle with the simultaneous deposition of a new cuticle. The acellular area between these two cuticles, which shows numerous disorganized microfibers and disrupted lamellae, may represent the partial digestion of the inner layers of the old cuticle. The separation of the inner lamellae and the compactness of the more peripheral lamellae of the old cuticle would support this interpretation. The possibility that at least part of the old cuticle could be reutilized in the formation of the new cuticle is an attractive hypothesis since it conserves the energy store of the encysted cells.

The observation that the homogenates from cysts maintained at 10° C demonstrate significant polyphenol oxidase activity also suggests that the encysted cells possess the ability to manufacture new cuticle. Such an ability would have important survival value since it would enable the organism to repair or replace damaged cuticle during encystment.

I am grateful to Mr. David Cargo, Chesapeake Biological Laboratory, Solomons, Maryland, for his cooperation and the use of his facilities for the collection of the organisms used in this study.

I would also like to thank Mrs. Dorrette Worrell and Mrs. Sandra Zane for expert technical assistance.

Summary

1. The podocyst cuticle of the medusa, *Chrysaora quinquecirrha* is composed of a chitin-protein complex tanned by phenolic substances as demonstrated by chromatographic and enzymatic analysis.

2. Electron micrographs reveal this material to be arranged as concentric layers which, in turn, are composed of microfibers.

3. Evidence is presented which suggests that the encysting cells retain the ability to manufacture new cuticle during encystment.

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INDUCTION AND TERMINATION OF PUPAL DIAPAUSE IN *SARCOPHAGA* (DIPTERA: SARCOPHAGIDAE)[1]

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Numerous studies on the bionomics of flies report the existence of a third-instar- or pupal diapause, but knowledge of the environmental factors regulating diapause is sparse, and information on the physiology of fly diapause is practically non-existent. In flesh flies of the genus *Sarcophaga*, experiments on diapause have been restricted to studies by Roubaud, 1922; Fraenkel and Hsiao, 1968a, 1968b; Denlinger, 1970, 1971b, 1972; Saunders, 1971; Vinogradova and Zinovjeva, 1971.

Diapause in *Sarcophaga* is most commonly observed in the young phanerocephalic pupa, a stage in which adult development has not been initiated. Occasional *S. bullata* pupae diapause in a slightly more advanced state, in which adult antennal discs are visible (Fraenkel and Hsiao, 1968a), and preliminary observations (William Downes, Michigan State University, personal communication) indicate that a larval diapause may also exist in several *Sarcophaga* species found in southern United States.

Inadvertent exposure of *S. argyrostoma* larvae to cold temperature and short daily photophase first revealed the importance of these factors in pupal diapause induction (Fraenkel and Hsiao, 1968a); however, larvae of *S. bullata* were not responsive to photoperiod. More recent experiments on pupal diapause in *S. crassipalpis* have demonstrated the extreme importance of the photoperiod received by embryos developing within the female's uterus (Denlinger, 1970, 1971b), and Saunders (1971) has reconfirmed a larval sensitivity to temperature and photoperiod in *S. argyrostoma*.

Although many of Roubaud's (1922) experiments were based on only one pupa, diapause did not appear to be terminated by high temperature or by mechanical or chemical means; however, chilling appeared to cause an early termination of diapause. An acceleration of diapause termination in *Sarcophaga* has also been accomplished with ecdysone injections (Fraenkel and Hsiao, 1968b).

The present investigation was carried out to define further the environmental parameters responsible for induction and termination of pupal diapause in flesh flies. Comparisons are made between different species and strains of *Sarcophaga*.

MATERIALS AND METHODS

Flies used in the experiments represent three species: *Sarcophaga argyrostoma* (Robineau-Desvoidy), *S. bullata* Parker, and *S. crassipalpis* Macquart. Laboratory cultures of *S. argyrostoma* and *S. bullata* were from the same strains used by Fraenkel and Hsiao (1968a). Wild strains of *S. bullata* started from single females collected in Champaign County, Illinois, and St. Louis County, Missouri, were also used. Data on the wild strains of *S. bullata* were collected during the first year of laboratory culture. The stock of *S. crassipalpis* came from progeny of three wild-caught females collected in Champaign County, Illinois, five years before this investigation. Stocks were maintained in an insectary at 26 ± 1° C in continuous light.

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[1] This paper is based on material contained in a thesis for the doctoral degree at the University of Illinois at Urbana-Champaign.

Environmental cabinets (Percival Refrigeration Co.) were used to maintain a temperature of 25 ± 0.5° C. Temperatures of 17 ± 1.0, 26 ± 1.0 and 28 ± 1.0° C were maintained in walk-in temperature cabinets. The vicinity of the experimental animals received a light intensity range of 10–45 lux during the photophase.

Groups of about 100 adult flies were kept in 1 ft square aluminum framed, screened cages (Cornell Equipment Co.). Sucrose and water were provided throughout adult life; pork liver was provided as a source of protein from the first day following adult eclosion until the seventh day.

All three species are ovoviviparous. Embryonic development occurs within a sac-like uterus, and, at 25° C, eggs hatch in the uterus by the eleventh day of female adult life. In the absence of liver, larvae are not deposited by the females. Collections were made by snipping off the tip of the female's abdomen on the eleventh day and extruding the larvae. Thus, a uniform starting time was achieved for progeny of different females.

The number of progeny produced in the first ovarian cycle differs among the species and strains examined. The mean numbers of progeny/female are as follows: *S. argyrostoma,* 30.2; *S. crassipalpis,* 61.7; *S. bullata* (lab), 55.6; *S. bullata* (Mo.), 96.4; *S. bullata* (Ill.), 103.4.

Larvae were reared on fresh pork liver homogenized in a Waring blender. Progeny of single females were reared in separate packets made of .001 gauge aluminum foil. Boats with a area of 8 cm × 4.5 cm were produced by forming a piece of foil 20 cm × 23 cm around a block of wood. Liver was weighed into each boat in order to provide about 0.5 g/larva. After addition of the larvae, the top of the packet was compressed and the excess aluminum foil was cut off, leaving a narrow slit open at the top. The packets containing the liver and larvae were placed on 1 cm sifted sawdust within a 16 oz squat container (Dixie Products, No. 2186-SE). Mass rearings of 400–600 larvae for the stock cultures were carried out in 11 cm × 9 cm foil boats. When the third instar larvae cease feeding they crawl out of the aluminum foil packets and pupariate in the sawdust. The rearing method serves to reduce the odor associated with the meat and has the advantage of allowing easy separation of pupae from the rearing medium.

The incidence of diapause is more consistent among different batches when small numbers of larvae such as the progeny of single females are reared together. In mass rearing the temperature in the microclimate produced by the liver and larvae rises many degrees above ambient temperature and thus may cause variation among batches. To minimize variation, all results are based on data collected from separate packets containing progeny of single females.

The incidence of diapause was determined by removing the anterior cap of the puparia to examine the pupae for the presence of imaginal development. In experiments reporting the time required for diapause termination, pupae were examined every 10 days. From knowledge of the time required for attainment of various post-diapause developmental stages, the date of the first visible signs of diapause termination (antennal spots observed by Fraenkel and Hsiao [1968a]) could easily be estimated. Although respiration studies show that breaking of diapause can be detected several days before the appearance of the first morphological signs of adult development (Denlinger, Willis, and Fraenkel, 1972), the day adult antennal discs became visible has been designated as the day of diapause termination.

RESULTS

Role of adult and larval photoperiod and temperature on diapause induction

The incidence of diapause observed under different adult and larval photoperiods and temperature is recorded in Table I. There are no significant differences in the effects of 8, 10 or 12 hour photophases; therefore, results from these photophases are combined, and are denoted in Table I as "short."

Diapause is averted in all species and strains if a long photophase or continuous light is provided for both the adult mothers and larval offspring at 25° C.

TABLE I

Influence of maternal adult and larval photoperiod and temperature on incidence of pupal diapause in Sarcophaga *species and strains*

Experimental conditions				Species and strains									
Maternal adults		Larvae		*S. argyrostoma*		*S. crassipalpis*		*S. bullata* (lab)		*S. bullata* (Mo.)		*S. bullata* (Ill.)	
Photoperiod	Temp.	Photoperiod	Temp.	No. pupae	% diapause	No. pupae	% diapause	No. pupae	% diapause	No. pupae	% diapause	No. pupae	% diapause
24:0	25° C	24:0	25° C	875	0	1089	0	743	0	449	0.2	1172	0
"	"	short	"	118	0	38	0	689	0	368	0	294	0
"	"	"	17	342	84.3	1138	4.0	220	0.4	311	11.6	478	32.6
17:7	"	17:7	25	—	—	872	0	—	—	1666	0	1618	0
"	"	short	"	—	—	170	1.2	—	—	492	0	426	0
short	"	24:0	"	240	0	1242	9.1	280	0	493	0	212	0
"	"	17:7	"	53	0	237	9.3	643	1.9	143	11.9	380	11.0
"	"	short	28	86	15.1	372	57.0	112	6.2	465	8.2	404	14.8
"	"	"	25	183	31.8	959	85.7	621	22.5	1502	87.3	2537	73.4
"	"	"	17	234	100	568	100	542	73.8	150	100	179	100

"Maternal" photoperiod is important for all of the flies; under the same larval conditions the diapause level is always higher following maternal exposure to short day. Thus, no diapause occurs when larvae are reared at short day and 25° C without short day exposure of the maternal adults, and larvae reared at 17° C have a higher diapause incidence when their mothers are exposed to short day. Maintaining short-day adults of *S. crassipalpis* and *S. bullata* (lab) at a higher temperature (28° C) does not appear to alter the incidence of diapause in short-day progeny reared at 17, 25 and 28° C (each $N \geq 287$ pupae).

The important role played by the adult photoperiod is supplemented with a reinforcing role of photoperiod acting on larvae reared at 25° C. If only the maternal adults receive a short daily photophase, the level of pupal diapause is low. The diapause level increases greatly with larval exposure to short day. Progeny of short day *S. bullata* (Ill.) adults reared in continuous darkness at 25° C ($N = 183$ pupae) show a great decrease in diapause incidence as compared to larvae reared at short day (3% in contrast to 73%).

The effect of photoperiod is influenced by the temperature at which the larvae are reared. Under experimental conditions in which only the larval rearing temperature is variable, the incidence of diapause increases with a decrease in temperature. Thus, the maximum diapause response for all flies occurs with a combination of short days for the adults and short days and low temperature (17° C) for the larvae.

There are differences between the species and strains of *Sarcophaga*. In the absence of adult short photoperiod, *S. argyrostoma* is more sensitive than the other species and strains to the larval diapause-inducing factors of short photoperiod and low temperature. The laboratory strain of *S. bullata* enters diapause at the lowest rate under each of the experimental conditions. Differences also exist between the two wild populations of *S. bullata*. When the entire life cycle is spent under a short daily photophase at 25° C, the level of diapause is greater for the strain collected in Missouri (see also Fig. 1).

Critical photoperiod

Samples of *S. bullata* from Missouri and Illinois were maintained throughout the life cycle at 25° C under different photoperiod regimens. Each point in Figure 1 represents the mean diapause incidence in the progeny of 12–14 females; 925–1666 pupae were produced by these females.

The transition between diapause induction and continuous development occurs abruptly at a daily photophase of 13½ hours for both populations. With a daily photophase shorter than the critical photoperiod, the diapause level is high. The unnatural conditions of less than an 8-hour photoperiod caused a decline in the diapause response of *S. bullata* (Ill.).

Effect of water content of larval medium on diapause induction

S. crassipalpis larvae taken from the same lot of adults maintained at 25° C-12 hour photophase were reared either on the standard medium of homogenized fresh pork liver or on a mixture of the medium + 10% water (40 g liver + 4 ml H_2O/progeny of 1 female). Larvae were reared at 25° C-12 hour photophase in the same incubator. Progeny reared on the standard medium produced an 86.1% ($N = 553$) incidence of pupal diapause; addition of 10% water to the medium produced a 95.7% ($N = 716$) incidence of diapause (Pearson $\chi^2 = 29.69$, d.f. = 1, $P < 0.01$).

Effect of adult protein deprivation on diapause

Adults of *S. argyrostoma,* a species capable of producing its first batch of progeny without protein, were maintained on a protein-free diet (Denlinger, 1971a); other flies from the same lot were provided protein. The incidence of diapause in the progeny of the two groups was compared (81.6% without protein, N = 136; 84.3% with protein, N = 186 pupae). The test failed to show a significant difference in progeny diapause level (Pearson $\chi^2 = 0.29$, d.f. = 1, $P > 0.50$).

Influence of sex on diapause induction

Comparison of the sex ratios in groups of *S. crassipalpis* representing various levels of diapause incidence shows a tendency for males to enter diapause at a higher rate than females. The sex ratio approaches 1 : 1 for batches representing 0% diapause (51% males, N = 993) and 100% diapause (48% males, N = 761).

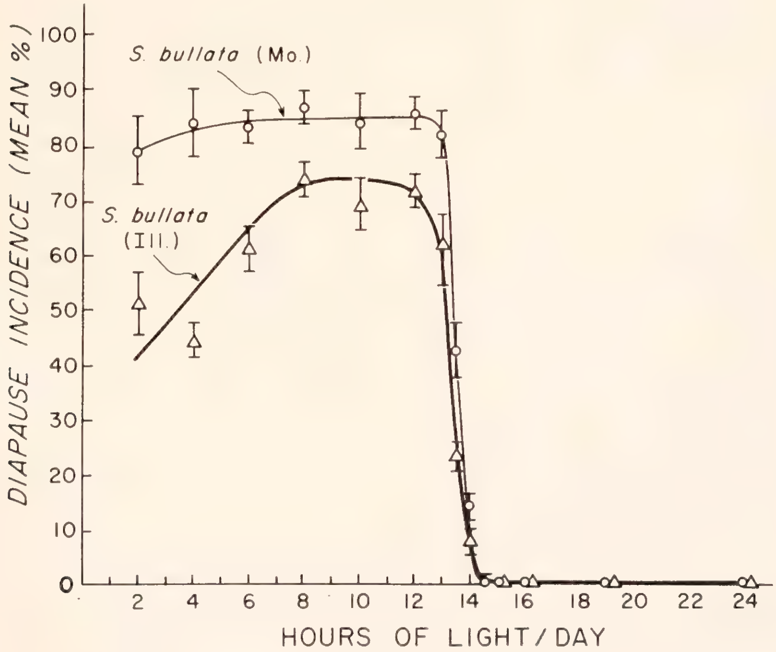

FIGURE 1. Critical photoperiod at 25° C in samples of wild populations of *Sarcophaga bullata* from Illinois and Missouri. Each point represents the mean (± S. E.) diapause incidence in progeny of 12–14 females (925–1666 pupae).

With partial diapause incidence the sex ratios of the diapause and non-diapause fractions deviate significantly from 1 : 1. In batches representing < 10% diapause, 87% of diapausing flies were males (N = 102). The tendency for males to enter diapause more readily than females is further exemplified by the sex ratio of flies developing without a diapause in batches representing > 90% diapause (25% males, N = 124). Observations on the other species of *Sarcophaga* have revealed a similar pattern.

Delay of pupariation in diapause-committed larvae

Flies reared under diapause-inducing conditions pupariated later and over a greater span of time than flies reared under non-diapause conditions. Adult flies originating from one mass-reared batch were maintained under either a short or long daily photophase at 25° C, and all larvae were maintained at a short photophase at 26° C. At 24 hour intervals following collection of the larvae, the rearing containers were examined, and the number of larvae having left the liver and the number having pupariated within the time interval were recorded. Pupae from diapausing lots were combined on the basis of the day of pupariation and were later examined to determine the incidence of diapause.

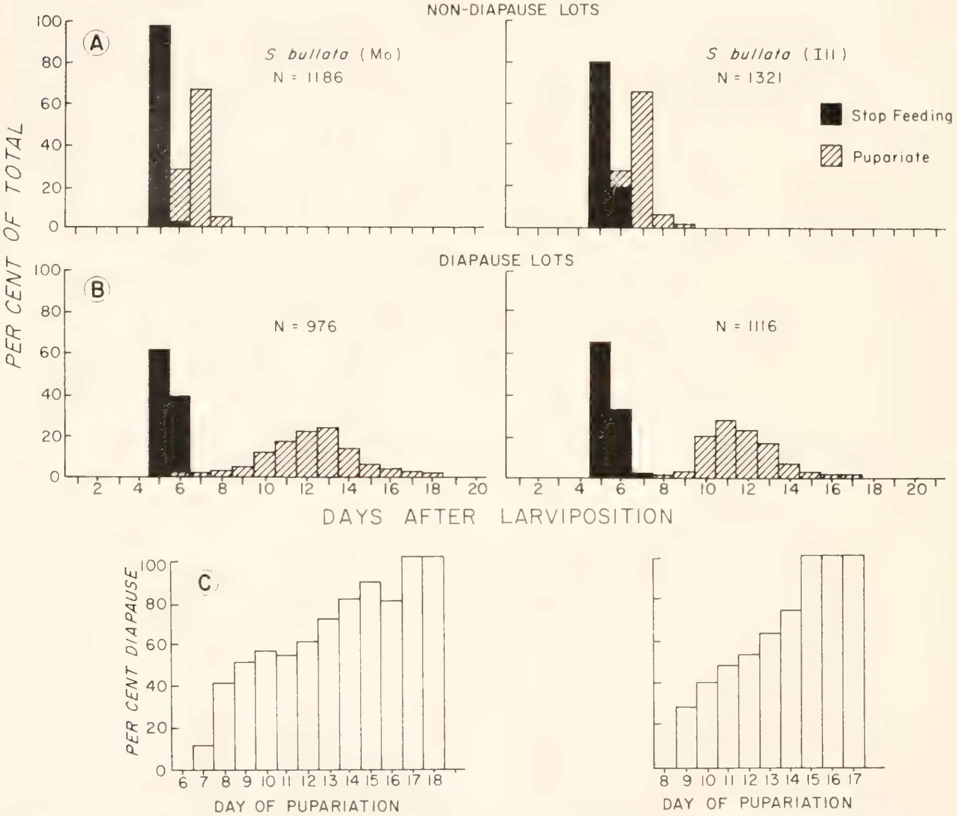

FIGURE 2. Delay of pupariation and correlation with incidence of pupal diapause in *Sarcophaga bullata* reared under diapause-inducing conditions. All bars are continuous to the base line.

Data for *S. bullata* (Mo.) and *S. bullata* (Ill.) are recorded in Figure 2. Larvae from both the non-diapausing (long-day) lots and diapausing (short-day) lots cease feeding and leave the liver at approximately the same time. In the non-diapausing lots (Fig. 2A), pupariation follows immediately; by the seventh day following larviposition, over 90% of the larvae have pupariated. In contrast, pupariation in diapausing lots (Fig. 2B) is delayed and occurs over a period of many days. Examination of pupae collected on each day revealed an increase in the incidence of diapause with an increase in the time of pupariation (Fig. 2C). Studies with *S. crassipalpis* and *S. argyrostoma* have shown a similar relationship of pupariation and diapause incidence; however, in these species the delay is not quite so long as that observed with the wild strains of *S. bullata.*

Effect of temperature on diapause termination

Pupae held under a daily 12-hour photophase were maintained throughout diapause at either 17 or 25° C; the time required for diapause termination at the two temperatures is recorded in Table II. A multiple regression analysis indicated a highly significant interaction of species and strains with temperature ($F = 351.4$, d.f. = 4 and 1109, $P < 0.0001$). Temperature, species and strains, and their interaction account for 88.2% of the variation in duration of diapause. Comparison of all possible pairs of means by the SNK test, using MS error (= 329.6), indicates that all differences are significant except those designated in Table II. The two species with insignificant differences in diapause duration at one temperature are sgnificantly different at the alternate temperature.

TABLE II

Duration of pupal diapause at 17 and 25° C for species and strains of Sarcophaga. *Means followed by the same letter are not significantly different at 5% level.*

Species	Diapause duration (days) at constant temperature				Minimum diapause duration (days) at combination of temperatures*	
	17° C		25° C		Estimated no. days at each temperature	Mean total days
	No. pupae	Mean ± S.E.	No. pupae	Mean ± S.E.	17°/25°	
S. argyrostoma	92	227.4 ± 1.3	52	91.7 ± 1.1	35/35	70
S. crassipalpis	146	117.9 ± 0.4^{a}	216	69.8 ± 0.7^{b}	20/20	40
S. bullata (lab)	105	61.3 ± 1.1	98	70.6 ± 1.4^{b}	20/20	40
S. bullata (Mo.)	84	117.4 ± 1.3^{a}	108	106.5 ± 1.6	45/35	80
S. bullata (Ill.)	153	174.1 ± 2.5	63	133.3 ± 2.7	55/40	95

* See text.

With the exception of the laboratory strain of *S. bullata,* all the flies show a decrease in the duration of diapause with an increase in temperature. This relationship is further exemplified in *S. crassipalpis,* which has a mean diapause duration of 56.6 ± 1.4 days ($N = 87$) at 28° C. In this species duration of diapause was regressed on temperature (at 17°, 25°, 28° C), with temperature coded as °C minus 25°. The analysis of variance of the regression demonstrates a highly significant, negative curvilinear relationship between diapause duration and temperature (linear component with $F = 1691.5$, d.f. = 1, M.S. = 277138, $P < 0.0001$; quadratic component with $F = 8.1$, d.f. = 1, M.S. = 1322, $P < 0.001$; Residual

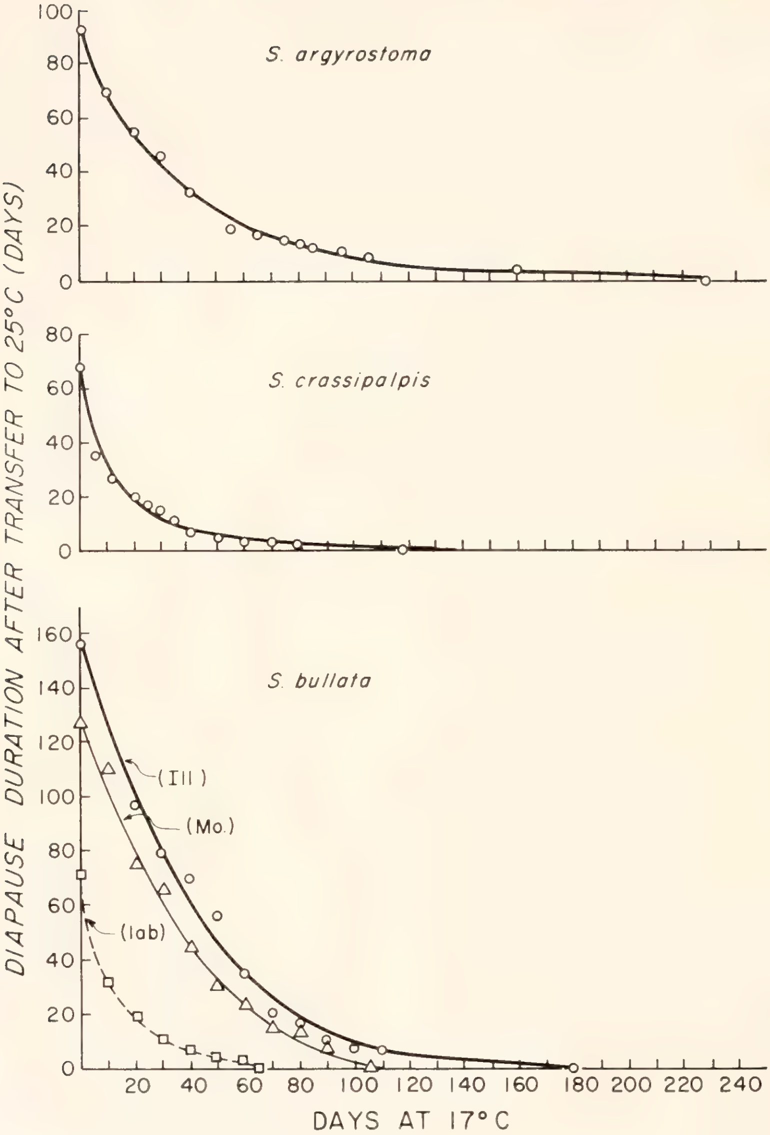

FIGURE 3. Duration of diapause in *Sarcophaga* species and strains at 25° C after various lengths of time at 17° C. Points on axes are from Table II; all other points represent the mean of 25–30 pupae.

d.f. = 446, M.S. = 164). The estimated regression equation is $\hat{Y} = 69.41 - 4.78X + 0.16X^2$, where X is coded temperature.

By providing diapausing pupae with a combination of 17 and 25° C, the duration of diapause decreases to a lower level than is observed with constant exposure to either temperature. Groups of 25–30 pupae were transferred from 17° to 25°

at 10 day intervals following pupariation; the mean number of days required to break diapause after transfer to 25° C is recorded in Figure 3. Points on the axes of the graphs were obtained from data on diapause duration at constant temperatures (Table II). The shape of the curve is similar for all of the species and strains although there are differences in the points of interception with the axes. The minimum number of days to break diapause has been estimated by calculating the point of the curve which is closest to the origin. The combinations of days which provide the minima are recorded in Table II. The duration of diapause can be reduced by 20–40 days by initial exposure to 17° C and subsequent transfer to 25° C.

Effect of non-temperature factors on diapause termination

To test the possibility that photophase influences the duration of diapause, lots of diapausing *S. crassipalpis* pupae at 25° C were divided into halves which were kept under a daily 12 hour photophase or transferred to a daily 17 hour photophase 10 days after pupariation. Of the 21 replications of the experiment performed, 3 replications (containing a total of 1044 pupae) were selected randomly for a multiple regression analysis incorporating terms for photophase, lots, and their interaction. Interaction was not significant and was dropped from the model. In the reduced model the effects of both lots and photophase were significant at levels of 0.1% and 5%, respectively. An increase of photophase from 12 to 17 hours increased the diapause duration 1.8 days. Lots and photophase together accounted for only 4.7% of the variation in duration of diapause and photophase alone for less than 1%. Even if the very slight difference in duration of diapause under the short and long photophases is not accounted for by an unassumed bias, the difference produces little biological effect.

The duration of diapause in diapausing *S. crassipalpis* pupae from lots with a low incidence of diapause (36.4%, N = 212 pupae) was compared with lots having a high incidence of diapause (94.2%, N = 248 pupae). The 2.3 day difference in latency (high > low) found to be significant with the Student's *t* test is not greater than can be attributed to the variation among lots.

DISCUSSION

Stimuli for induction

Adverse winter conditions in the temperate regions have channeled the evolution of a pupal diapause in flesh flies of the genus *Sarcophaga*. As has been demonstrated in many invertebrates (reviews by Lees, 1955; de Wilde, 1962; Danilevskii, 1965; Beck, 1968), environmental cues of short photoperiod and low temperature are primarilly responsible for programming diapause in flesh flies.

Fraenkel and Hsiao's (1968a) and Saunders' (1971) experiments with larvae of *S. argyrostoma* have demonstrated the role played by short photoperiod and low temperature on diapause determination; larval photoperiod was not shown to influence diapause in *S. bullata*. My experiments confirm their results, but supplement their data with information on the extreme importance of maternal adult photoperiod among all *Sarcophaga* examined. If only the larvae receive a short daily photophase at 25° C, the pupae will not enter diapause. If adults as well as larvae receive a short-day stimulus, a moderate incidence of pupal diapause occurs, even in *S. bullata.* Maximum diapause response occurs when adult and larval short-day stimuli are supplemented with larval exposure to 17° C. Investigation of the role played by the adult photoperiod has shown that the photoperiodic stimulus acts directly on the embryos developing within the uterus of the ovoviviparous females (Denlinger, 1970, 1971b).

At 25° C, photoperiods longer than 13½ hours permit continuous development in populations of *S. bullata* from Missouri (38°30′ N, 90°30′ W) and Illinois (40°15′ N, 88°15′ W). Shorter photoperiods which occur naturally in Missouri and Illinois are diapause-inducing. In *S. bullata* (Ill.) unnaturally short photoperiods produce a gradual decline in diapause response. A laboratory strain of *S. argyrostoma* examined by Saunders (1971) has a critical photoperiod of 13½–14 hours at 15° and 20° C. Differences in the critical photoperiods of populations from different geographic regions are commonly observed (Danilevskii, 1965); however, no such difference has been observed across the small latitudinal distance of 1°45′ for *S. bullata.*

Food quality and quantity may exert an influence on diapause in some insects. The physiological or ecological meaning behind the 10% increase in diapause with an increase in the moisture content of the larval medium in *S. crassipalpis* remains unknown. Literature on the role of water for diapause termination is abundant (Beck, 1968), but the role of water in diapause induction is less well documented. An opposite effect of moisture content was observed in *Lucilia sericata;* dry meat produced a greater incidence of third-instar diapause (Cousin, 1932; Mellanby, 1938). Diapause in the progeny of *S. argyrostoma,* a species capable of producing viable eggs in the absence of an adult protein meal (Denlinger, 1971a), was not affected by adult protein deprivation. It is not surprising that maternal nutrition does not influence diapause since there is no evidence that the female passes nutritive material to the embryos within her uterus, as was suggested by Cholodkovsky (1908); the fact that embryos can be cultured *in vitro* (Denlinger, 1971b) emphasizes the physiological independence of the embryos.

Sexual differences in tendency to enter diapause are especially apparent from examination of lots exhibiting a partial diapause response. Males of *Sarcophaga* enter diapause at a higher rate than females. *Lucilia caesar,* a species with a larval diapause, shows a similar tendency (Ring, 1971). The sexual differences in *Sarcophaga* imply a slightly different threshold for photoperiod and temperature response. Receiving a short-day stimulus for only the embryonic period (produces 10% diapause) is apparently insufficient for diapause induction in most females. A temperature threshold appears to be involved also since at short day-25° C (produces 85% diapause) three times as many of the non-diapausing flies are females; lowering the temperature to 17° C produces 100% diapause and an even sex ratio in the diapausing pupae.

Characteristics of diapause-committed larvae

Induction of pupal diapause in *Sarcophaga* is strongly correlated with another developmental feature, delay of pupariation. There is a general tendency for prediapause development to be slower than nondiapause development (Beck, 1968). This retardation appears to be exemplified by *Sarcophaga* because pupariation in diapause-committed lots of larvae occurs several days after pupariation in non-diapause lots. Within a given batch of short-day larvae the incidence of diapause increases directly with the time of pupariation. The retardation of development is not evenly distributed throughout larval life, but occurs after feeding has ceased. During the post-feeding period larvae wander in search of a dark, dry area for pupariation; the crop is gradually cleared during this time.

The reason behind such a delay is unknown. An adaptive advantage may be afforded a diapause-destined larva if it has more time to find a suitable hibernaculum. On a physiological level this phenomenon may be pointing to an early hormonal difference between larvae which will enter pupal diapause and those which will complete development immediately. The period of delay in the post-fed third instar occurs at the same developmental stage as the larval diapause in numerous other Diptera. Perhaps the longer larval life in diapause-committed *Sarcophaga* offers an evolutionary clue to the similarity of larval and pupal diapause.

Experimentally, pupariation can be delayed in *Sarcophaga* by exposure of the full-grown larvae to moisture (Evans, 1935; Ohtaki, Milkman and Williams, 1968; Zdarek and Fraenkel, 1970) and by larval injections of juvenile hormone (Srivastava and Gilbert, 1969). However, delaying pupariation by keeping full-grown larvae wet for several days is not sufficient to induce pupal diapause (Denlinger and Zdarek, unpublished observation). Arrest during pharate adult development can be brought about with larval injections of juvenile hormone in *S. bullata* (Srivastava and Gilbert, 1969). Perhaps these results may be interpreted as a diapause-like event. The fact that the arrest occurs at different life stages than is observed with naturally occurring diapause may not be too critical, since occasional *S. bullata* pupae also diapause in a slightly later developmental stage.

Lowering the larval rearing temperature delays pupariation, and concurrently there is an increase in the incidence of diapause (Saunders, 1971). Saunders attributes the increase in diapause not to a direct temperature effect but to an increase in the number of days of exposure to the short-day regimen. This hypothesis does not explain a difference in the time of pupariation between larvae from long-day adults and larvae from short-day adults which are reared side by side at 26° C-12 hour photophase as was done in this investigation. A comparison of these two groups of larvae reared at the same temperature indicates that the larva's commitment to pupal diapause is the cause of the delay and not the result of it. That larvae which will enter diapause receive additional light cycles due to a delay in time of pupariation is inconsequential to the induction of diapause in a species such as *S. crassipalpis* where the late larval life is unimportant for diapause determination (Denlinger, 1971b).

Stimuli for termination

The effect of "chilling" reported in *Sarcophaga* by Roubaud (1922) and Fraenkel and Hsiao (1968a) is confirmed by the persent observation on the acceleration of diapause termination by transferring pupae from 17 to 25° C. Physiologically the effect of change in temperature may result from interaction of various biochemical processes having different temperature optima.

The observed relationship between temperature and diapause termination provides interesting insights on the nature of diapause in *Sarcophaga*. Unlike many species which cannot terminate diapause at a constant temperature, *Sarcophaga* pupae entering diapause at constant temperatures ranging from 13.5° C (Fraenkel and Hsiao, 1968a) to 28° C can utlimately terminate diapause at that same temperature. The rate of completion for the physiological processes of diapause accelerates with temperature. Although such a relationship is common for most biological processes, Williams (1956) found no apparent rate change for pupal diapause termination in *Hyalophora cecropia* held at constant temperatures ranging between 10–25° C.

When diapausing Cecropia silkworms are "chilled" at constant temperatures ranging from 2½ to 20° C and then transferred to 25° C, the time required for initiation of adult development is reported to remain constant with the first few weeks of chilling and then to drop off precipitously (Williams, 1956). The initial plateau of the curve, a period which Williams attributes to a threshold reaction for reactivation of the brain, is not observed when *Sarcophaga* pupae are transferred to 25° C after various periods of "chilling" at 17° C. In all five of the species and strains of *Sarcophaga* examined, the duration of diapause begins to fall off immediately with time. Differences in the diapause intensity of the two insect groups or the fact that the Cecropia pupae received ten weeks of 25° C prior to the chilling experiments may account for the observed differences. The ability of ecdysone to terminate pupal diapause in both *H. cecropia* (Williams, 1946) and *Sarcophaga* (Fraenkel and Hsiao, 1968b) implies a similar hormonal basis for the diapause state.

Dependency on temperature rather than photoperiod as a cue for the termination of diapause is ecologically appropriate for *Sarcophaga* since the pupae are normally buried under the surface of the soil. In this situation photoperiod would not provide a readily accessible seasonal cue, whereas soil temperature could provide an accurate assessment of the advent of the favorable season in temperate regions.

It was thought that the duration of diapause may vary between lots with a low and high incidence of diapause. Such a difference in the intensity of diapause is not apparent. On an individual basis diapause is not a graded response but an "all or none" phenomenon.

Species and strain differences

Experiments with *S. argyrostoma*, *S. crassipalpis*, and three strains of *S. bullata* demonstrate the variability of the diapause characteristics among species and strains. Unlike the other flies, *S. argyrostoma* responds with a high incidence of pupal diapause if the larvae are reared at 17° C-12 hour photophase without an adult short-day stimulus; the role of adult photoperiod is more important in other species. In most cases the direction of response to temperature and photoperiod observed is the same but differs in degree. Differences in the duration of diapause exist among the *Sarcophaga*; a difference exists between the wild *S. bullata* strains from Illinois and Missouri, and a very pronounced difference exists between the wild strains and the laboratory strain of the same species. Many years of selection and inbreeding in the laboratory under continuous light and constant temperature may account for the aberrance of this strain. The results with the laboratory strain of *S. bullata* suggest the need for caution in projecting to the natural world results obtained from highly inbred laboratory animals.

I am indebted to Dr. W. Downes of Michigan State University for supplying *S. crassipalpis* and the wild strain of *S. bullata* from Missouri; his knowledge of *Sarcophaga* and suggestions of rearing techniques were invaluable. I am also grateful to Drs. G. S. Fraenkel and J. H. Willis for their helpful suggestions throughout the investigation, to Dr. R. B. Selander for his comments on the manuscript and assistance with the statistical analysis, and to Mrs. Alice Prickett for drawing the figures in the manuscript. The research was supported in part by a grant from NIH (E-533) to Dr. G. S. Fraenkel.

SUMMARY

1. In temperate regions species of *Sarcophaga* overwinter in pupal diapause. The environmental control of diapause is investigated in *Sarcophaga argyrostoma, S. crassipalpis,* and three strains of *S. bullata.* Environmental cues of daylength and temperature, water content of the larval medium, and the sex of the animal determine the induction of diapause. Termination of diapause is temperature dependent.

2. Daylength is of primary importance for induction. Diapause is completely averted when adult mothers and larvae are maintained under a long daily photophase or continuous light at 25° C. Short-day exposure of the adults and larvae at 25° induces a high incidence of diapause. However, if short-day is received by only the larvae, diapause is absent, and adult short-day without subsequent larval short-day produces a low diapause incidence. The maximum diapause response is observed when adults are maintained under a short daily photophase at 25°, and larvae are reared at a short daily photophase at 17°.

3. Critical daylength for wild populations of *S. bullata* from Illinois and Missouri is 13½ hours at 25°.

4. A decrease in larval temperature from 28° to 17° C increases the incidence of pupal diapause in animals reared at short-day.

5. Addition of 10% water to larval medium increases diapause incidence 10%.

6. Males enter diapause at a higher rate than females. Sex ratio approaches 1 : 1 in pupae representing 0 and 100% diapause, but lots showing a partial diapause response have a higher percentage of males than females in diapause.

7. Larvae reared under diapause-inducing conditions pupariate over a period of days 3–4 times greater than larvae reared under non-diapause conditions; mean day of pupariation is 4–5 days later in diapause batches. The incidence of pupal diapause increases with an increase in the delay of pupariation.

8. Photoperiod is ineffective in terminating diapause.

9. At constant temperatures the duration of diapause decreases with an increase in temperature. A combination of 17° and 25° provides a shorter diapause than constant exposure to either temperature.

10. Significant differences are found in the diapause responses observed with

different species of *Sarcophaga;* wild strains of *S. bullata* also differ from each other and differ greatly from a lab strain of the same species.

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OSMOREGULATION IN THREE MOLLUSCS: *ACANTHOCHITONA DISCREPANS* (BROWN), *GLYCYMERIS GLYCYMERIS* (L.) AND *MYTILUS EDULIS* (L.).

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Molluscs certainly constitute one of the most representative phyla of the littoral area, inasmuch as they have often been chosen as landmarks in the definition of the shore levels. In these biotopes, molluscs withstand frequent and rapid changes in the osmotic pressure of their environmental medium. The euryhalinity of aquatic molluscs has been the subject of many investigations (for review see Robertson, 1964; Schoffeniels and Gilles, 1970a) and it is classically assumed that, in these species, this is the cell which has to cope with the osmotic stress. Marine molluscs are indeed considered as poecilosmotic animals having no power of anisomotic extracellular regulation. However, if the blood of the periwinkle *Littorina* is isosmotic with the medium in salinities down to 17‰, it becomes hyperosmotic in more diluted media, the mean blood △ being 1.06° C against 0.48° C for the medium (Todd, 1964). In the same way, a mean blood △ of 0.74° can be recorded for specimens of the bivalve *Scrobicularia plana* acclimated to a medium of 0.59° C (Freeman and Rigler, 1957) and the salinity inside the mantle cavity of *Mytilus* can be 24‰ when the salinity outside is only 7‰ (Milne, 1940). Since the hyperosmotic state observed in marine gastropods submitted to an osmotic stress is known to be due to the ability of these animals to close tightly their internal cavity, one wonders, therefore, if the hyperosmotic state reported in *Mytilus* or *Scrobicularia* is not due, at least partially, to such a "shell-closing" mechanism.

This study deals, therefore, with the incidence of the "shell-closing" mechanism on the ability of some bivalve molluscs to withstand an osmotic stress. Moreover, since amino acids play a part in the regulation of the cellular osmotic pressure in molluscs (for review, see Schoffeniels and Gilles, 1970a), the changes in the intracellular amino-acid pool during acclimation of the studied species to various media have also been considered.

MATERIALS AND METHODS

The studied species—*Glycymeris glycymeris* (L.), *Mytilus edulis* (L.) and *Acanthochitona discrepans* (Brown)—were brought from the Roscoff Marine Laboratory (France) and allowed to recover in the laboratory for two weeks

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Permanent address: Department of Biochemistry, University of Liège, 4000-Liège, Belgium before use. All the experiments have been carried out at 17° C. In a first series of experiments, lots of 75 to 100 animals of each species were acclimated from sea water to different media (50%–25% sea water or fresh water) either directly or by gradual steps. During the time course of the acclimation, samples of the body fluids have been taken for osmotic pressure and ionic concentration determinations. Perivisceral fluid samples have been taken in the perivisceral cavity while maintaining the valves ajar. Blood samples were collected by direct puncture in the pericardiac cavity. Osmotic pressure measurements of these fluids have been achieved with a mechrolab osmometer. Measurements of Na^+ and K^+ concentrations have been run with a flame photometer and Cl^- concentration has been estimated by potentiometric titration.

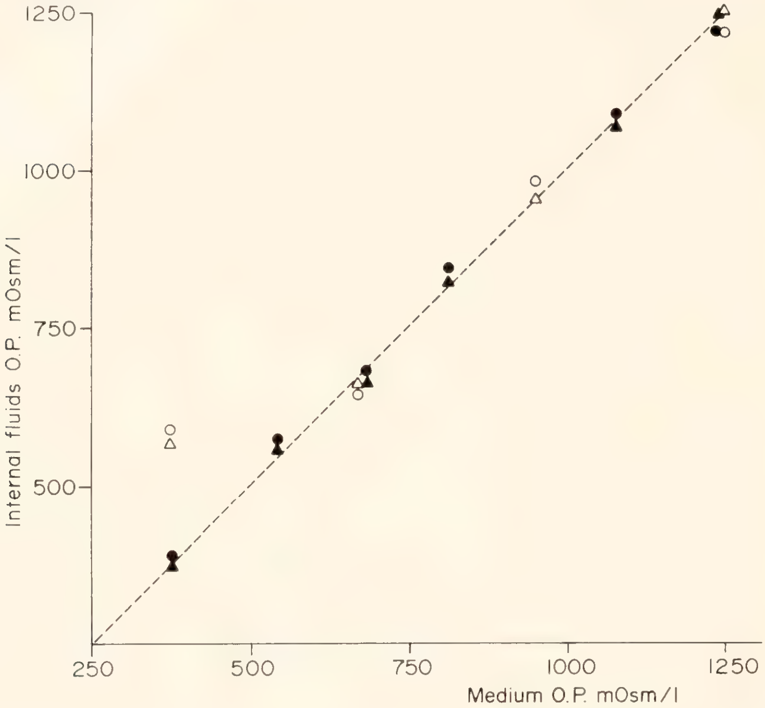

FIGURE 1. Changes in the osmotic pressure of the blood (○) and of the perivisceral fluid (△) of *Glycymeris glycymeris* during rapid (open data points, ○, △) or slow (closed data points, ●, ▲) acclimatization to diluted media. The osmotic pressure is given in milliosmoles/l; dashed line: isosmoticity line. Points are the average of 5 determinations, each one being performed on a sample obtained by pooling the blood or the perivisceral fluid of 4 to 10 animals. The standard deviation never exceeds 4.8%.

In another series of experiments, the molluscs were maintained either in sea water or in 50% sea water for theree weeks. Samples of adductor muscle or foot muscle were then taken for amino-acid and water content determinations. The concentration of the free intracellular amino acids was determined with a

"Technicon autoanalyzer" following the method previously described (Gilles and Schoffeniels, 1968). The water content was estimated by the fresh weight-dry weight technique.

RESULTS

(*A*) *Body fluids osmotic and ionic concentrations*

During rapid acclimatization of *Glycymeris glycymeris* to media of various salinities, the blood and the perivisceral fluid of the bivalve remain in osmotic equilibrium with the environmental medium down to salinities of 50% sea water. However, a hyperosmotic state is observed in both blood and perivisceral fluid when *Glycymeris* is acclimated to 25% sea water (Fig. 1). In these experiments, the animals remained two days at each dilution step. If the animals are maintained one week in each dilution, the hyperosmotic state observed in low salinities is no longer recorded and both blood and perivisceral fluid are in osmotic equilibrium with the external medium (Fig. 1). We have, therefore, undertaken experiments attempting to show the effect of the time of acclimatization to various media on the ionic composition of the blood and the perivisceral fluid of various molluscs. The results given in Figures 2 to 5 show the modification of the

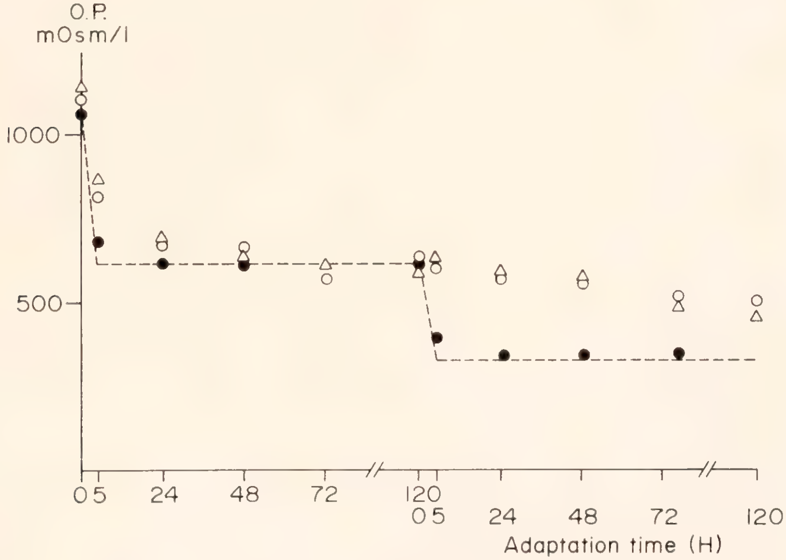

FIGURE 2. Changes in the osmotic pressure of the blood (○) and of the perivisceral fluid (△) of *Glycymeris glycymeris* (open data points, ○, △) or *Acanthochitona discrepans* (closed data point, ●) during the time course of acclimatization to diluted media. The osmotic pressure is given in milliosmoles/l; dashed line: external medium osmotic pressure. Points are the average of 5 determinations, each one being performed on a sample obtained by pooling the blood or the perivisceral fluid of 4 to 10 animals. The standard deviation never exceeds 4.8%.

osmotic pressure as well as of the Na, K and Cl concentration of the blood and the perivisceral fluid during the time course of acclimation of *Glycymeris* and *Acanthochitona* to 50 and 25% sea water. At each dilution tested, the Na and Cl concentrations as well as the osmotic pressure of the blood of *Glycymeris* follow the one of the perivisceral fluid. The osmotic pressure and the Na and Cl concentrations of this last fluid stay, however, higher than the one of the external medium for at least 72 hours (Figs. 2, 3 and 5). The perivisceral fluid reaches an isosmotic equilibrium with the environmental medium only after 120 hours. The changes in the perivisceral fluid K concentration follows the same pattern. However, it should be noted that the blood K concentration is regulated at the value it has in the blood of sea-water *Glycymeris* (Fig. 4).

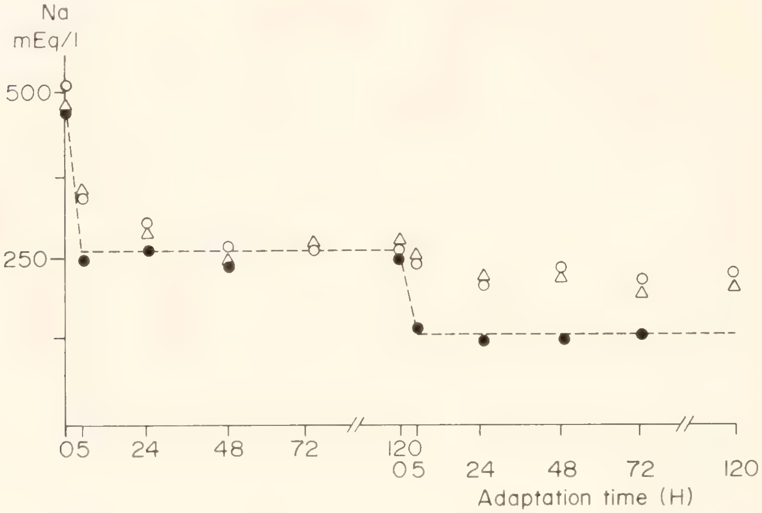

FIGURE 3. Changes in the Na concentration of the blood (○) and of the perivisceral fluid (△) of *Glycymeris glycymeris* (open data points, ○, △) or *Acanthochitona discrepans* (closed data point, ●) during the time course of acclimatization to diluted media. The Na concentration is given in mEq/l; dashed line: Na concentration of the external medium. Points are the average of 5 determinations, each one being performed on a sample obtained by pooling the blood or the perivisceral fluid of 4 to 10 animals. The standard deviation never exceeds 4.8%.

The results obtained with the euryhaline intertidal bivalve *Mytilus edulis* are similar to the ones described above for *Glycymeris.* In the case of *Mytilus,* indeed, the blood osmotic pressure remains similar to that measured in the perivisceral fluid. At high dilutions of the external medium (25% sea water and fresh water), the perivisceral fluid remains hyperosmotic to the environmental medium at least for 96 hours (Fig. 6). This is at variance with what happens in *Acanthochitona discrepans* where the blood always stays in isotonic equilibrium with the external medium (Figs. 2, 3 and 5). However, as it is the case for *Glycymeris,* the blood K concentration is regulated at the value it has in the blood of sea water acclimated animals (Fig. 4).

(B) *Intracellular free amino-acid pool*

As shown in Table I, the concentration of the amino acids is higher in the adductor muscle of *Mytilus edulis* than in the adductor muscle of *Glycymeris glycymeris* or in the foot muscle of *Acanthochitona discrepans.* Moreover, the amino-acid content decreases during the acclimation of these species from sea water to 50% sea water. This decrease mainly affects alanine, aspartic acid, glutamic acid, glycine, proline, serine and the amine, taurine. During hypoosmotic stress, the water content of the muscle increases by 7.8 to 10.6%. However, this increase cannot account for the reduction in the amino-acid and taurine content observed during the acclimatization to the diluted medium (Table I).

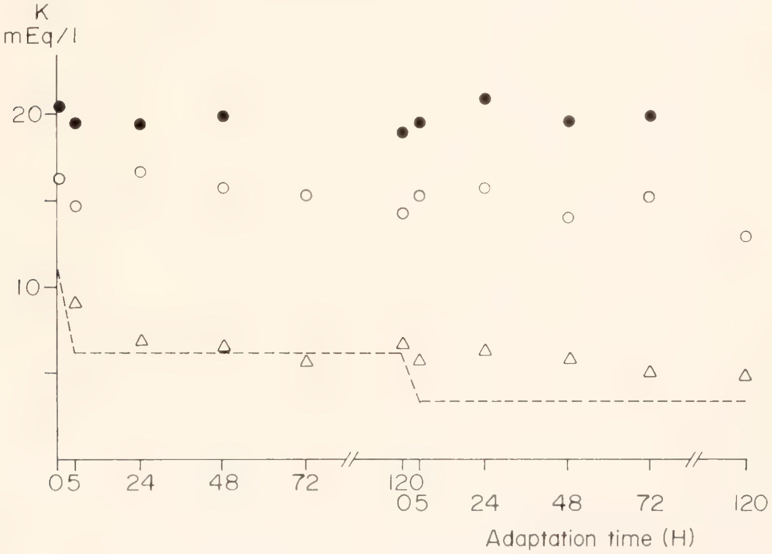

FIGURE 4. Changes in K concentration in the blood (○) and in the perivisceral fluid (△) of *Glycymeris glycymeris* (open data points, ○, △) or *Acanthochitona discrepans* (closed data point, ●) during the time course of acclimatization to diluted media. The K concentration is given in mEq/l; dashed line: K concentration of the external medium. Points are the average of 5 determinations, each one being performed on a sample obtained by pooling the blood or the perivisceral fluid of 4 to 10 animals. The standard deviation never exceeds 4.8%.

DISCUSSION

A hyperosmotic state can be observed in the body fluids of the bivalve mollusc *Glycymeris glycymeris* when subjected to a rapid acclimatization to 25% sea water. In these experiments, the animals remain for two days at each dilution step. If the animals are now maintained for one week in each medium, the hyperosmotic state observed in low salinities during rapid acclimatization is no more recorded and both the perivisceral fluid and the blood remain in osmotic equilibrium with the external medium. These results suggest that the hyperosmotic state observed during the rapid acclimatization of specimens of *Glycymeris* to diluted media is not due to a mechanism of anisosmotic regulation but rather to a mechanism attempting to isolate the animal from the external medium. Moreover, this mechanism appears to be effective only during a short period of time. We have, therefore, undertaken experiments in order to show the effect of the time of acclimatization to a diluted medium on the ionic composition of the body fluids of various molluscs. For these experiments, we have chosen two bivalves: *Glycymeris glycymeris* and *Mytilus edulis.* These species can isolate themselves from the environmental medium by closing tightly their valves. We have also used the Polyplacophora *Acanthochitona discrepans* which does not have such a "shell-closing" mechanism.

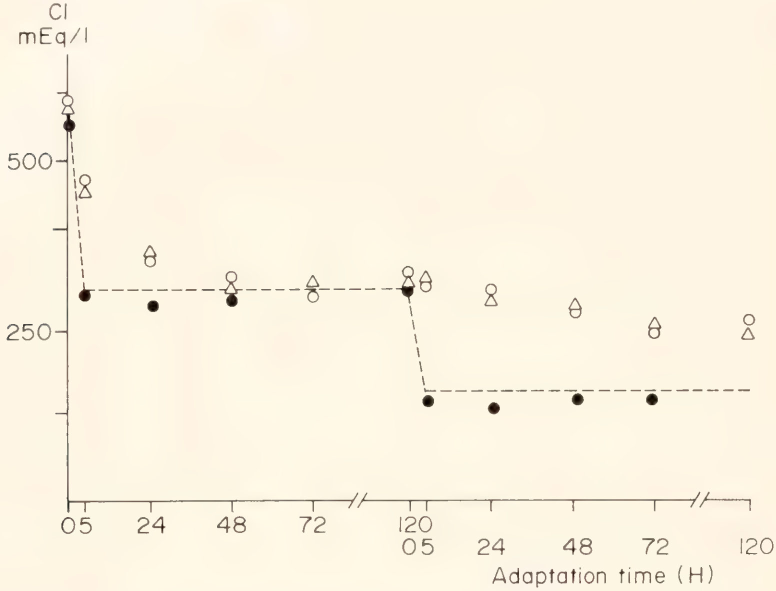

FIGURE 5. Changes in the Cl concentration of the blood (○) and of the perivisceral fluid (△) of *Glycymeris glycymeris* (open data points, ○, △) or *Acanthochitona discrepans* (closed data point, ●) during the time course of the acclimatization to diluted media. The Cl concentration is given in mEq/l; dashed line: Cl concentration of the external medium. Points are the average of 5 determinations, each one being performed on a sample obtained by pooling the blood or the perivisceral fluid of 4 to 10 animals. The standard deviation never exceeds 4.8%.

When the bivalves *Glycymeris* or *Mytilus* are submitted to hypoosmotic stress, the blood stays hyperosmotic to the external medium for at least 96 hours after the beginning of the acclimatization (Figs. 2 and 6). It has, however, to be noted that the blood, even when hyperosmotic to the external medium always remains isosmotic to the perivisceral fluid. On the other hand, the transitory hyperosmotic state observed in the blood of the bivalves when acclimated to a dilute medium is not recorded during the acclimatization of the Polyplacophora *Acanthochitona.* In this species indeed, which does not have a perivisceral fluid, the blood always remains in osomotic equilibrium with the surrounding medium.

The isosmotic equilibrium is reached immediately after the beginning of the acclimatization. It can, therefore, be concluded that the three species studied do not have any anisosmotic regulation mechanism since the blood always remains in osmotic equilibrium with its surrounding fluid. Moreover, the transitory hyperosmotic state observed in the blood of the two bivalves studied appears to be due to the fact that the perivisceral fluid is transitorily maintained hyperosmotic to the external medium. The same kind of results has been obtained with the gastropods *Littorina saxatilis* (Avens and Sleigh, 1965), *Siphonaria pectinata* (McAlister and Fisher, 1968) and *Littorina littorea*, *Purpura lapillus* and *Patella vulgata* (Hoyaux and Jeuniaux, in preparation). These last authors, moreover, demonstrate that the disequilibrium between the perivisceral fluid and the surrounding medium disappears if the operculum of *Littorina* is removed.

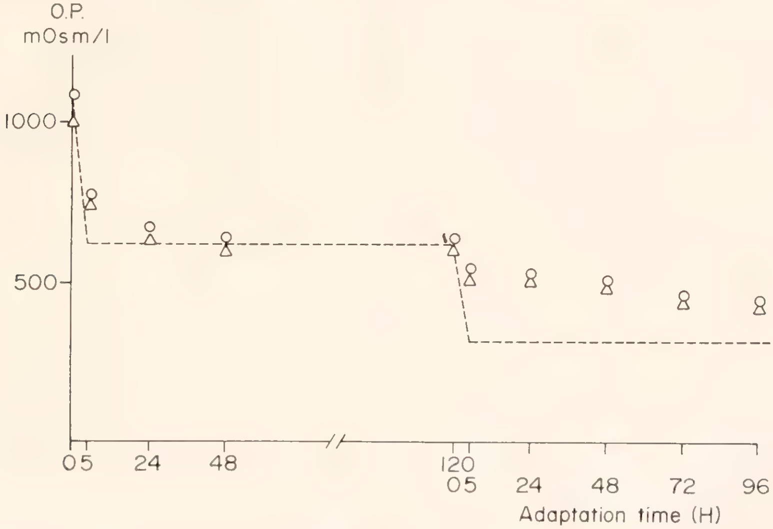

FIGURE 6. Changes in the osmotic pressure of the blood (○) and of the perivisceral fluid (△) of *Mytilus edulis* during the time course of acclimatization to diluted media. The osmotic pressure is given in milliosmoles/l; dashed line: external medium osmotic pressure. Points are the average of 5 determinations, each one being performed on a sample obtained by pooling the blood or the perivisceral fluid of 4 to 10 animals. The standard deviation never exceeds 4.8%.

It appears, thus, that some molluscs may be helped in withstanding a sudden osmotic stress by isolating themselves from the external medium. The temporary hyperosmotic state so obtained, which has been sometimes interpreted as the effect of an osmotic regulation in diluted media (see Todd, 1964) is achieved by various processes. Bivalves such as *Mytilus* or *Glycymeris* close their valves tightly, gastropods with an operculum such as *Littorina* or *Purpura* retract themselves strongly in their shell and *Patella* or *Siphonaria* adhere firmly to the rock. Such a behavior appears to be of considerable importance in the survival of these animals under temporary adverse conditions as is often the case in the intertidal or estuarine environment. This "shell-closing" mechanism can only help the animal

TABLE I

Amino-acid concentration in the muscle of Glycymeris glycymeris, Mytilus edulis *and* Acanthochitona discrepans *adapted to sea water or to 50% sea water. The concentration of the various compounds is given in μM/g wet weight tissue, tr: traces; —: not determined*

Amino acids	*Glycymeris glycymeris* adductor muscle		*Acanthochitona discrepans* foot muscle		*Mytilus edulis* adductor muscle	
	Sea water	50% Sea water	Sea water	50% Sea water	Sea water	50% Sea water
Alanine	7.60	7.10	1.80	1.70	28.07	8.77
Arginine	6.70	6.80	3.40	3.10	—	—
Aspartic acid	9.60	9.70	1.50	1.00	11.25	2.01
Glutamic acid	5.40	4.20	4.00	4.70	5.00	1.03
Glycine	2.10	1.30	0.60	0.70	132.69	58.92
Histidine	0.70	0.20	tr.	tr.	0.58	0.72
Isoleucine	0.20	0.10	tr.	0.10	0.90	0.35
Leucine	0.20	0.20	tr.	0.20	1.57	0.53
Lysine	0.60	0.70	0.30	0.40	1.09	0.72
Phenylalanine	0.80	0.05	tr.	0.10	0.60	0.40
Proline	tr.	tr.	0.40	0.40	45.65	10.95
Serine	3.40	1.40	0.70	0.90	2.86	3.17
Threonine	—	—	0.40	0.40	3.07	1.21
Tyrosine	tr.	tr.	0.10	0.10	0.90	0.58
Valine	—	—	0.20	0.20	2.24	0.75
Taurine	47.10	40.30	17.83	15.24	20.50	9.21
Water content	73.40	81.20	75.10	82.60	71.62	77.26
Osmotic pressure due to amino acids*	56.46	43.45	19.83	18.83	366.49	129.46
Osmotic pressure due to taurine*	71.30	55.14	26.35	20.48	31.77	13.23
Osmotic pressure of the external medium**	1180	573	1180	573	1180	573

* The osmotic pressure due to the various compounds is given in milliosmoles/kg tissue water (Δ of a molar solution of amino acids is −1.86° C—Prosser, Bishop, Brown, Kahn and Wulff, 1950).

** The osmotic pressure of the external medium is given in milliosmoles/l.

to wait for better conditions during a relatively short period of time and, at any rate, it cannot contribute to the osmotic regulation observed in molluscs during modification of the salinity of their environmental medium.

Although the blood of the species we have studied always remains in osmotic equilibrium with its surrounding fluid, the results we have obtained demonstrate that these species are capable of ionic regulation. Indeed, if Na and Cl concentrations are generally close to those of the surrounding fluid, the blood K concentration is regulated at the value it has in the blood of sea water animals (Fig. 4). This regulation of the blood K level has also been observed by Hoyaux and Jeuniaux (in preparation) when working with *Patella*, *Littorina* and *Purpura*. It is, however, impossible so far to determine if the maintenance of the potassium concentration at a given level is important or not in the osmoregulation process. Further studies are needed to bring some light on this problem.

The results discussed so far show that the studied molluscs have practically no anisosmotic regulation power. One can, therefore, consider that during the acclimatization of these species to a dilute medium, it is the cells which will have to cope with the osmotic stress. It has been shown by Florkin and co-workers that in molluscs, as well as in other euryhaline invertebrates, aminoacids play a part in the cellular osmotic regulation process. As a matter of fact, the concentration of the free amino acids is higher in the tissue of an euryhaline invertebrate when it is acclimated to sea water than when it is acclimated to a diluted medium (for review see Florkin, 1962, 1966; Florkin and Schoffeniels, 1969; Schoffeniels and Gilles, 1970a, 1970b). The results listed in Table I show that in the species we have used, the concentration of the amino acids and taurine is higher in the muscle of sea water acclimated animals than in the same tissue taken from animals acclimated to 50% sea water. The data we have obtained with *Mytilus* are in agreement with those given previously by Bricteux-Gregoire, Duchateau-Bosson, Jeuniaux and Florkin, (1964). On the other hand, the changes observed in the amino-acid concentration are greater than those which could be expected from the increase in the tissue water content. One should, therefore, consider these modifications as being the result of an active regulation process.

When comparing the changes in the concentration of individual amino acids in the three species studied, it is apparent that the changes in the amino-acid concentration do not follow a single general pattern. As a matter of fact, alanine acts as an osmotic effector only in *Mytilus* while the concentration of glycine decreases during the acclimatization to 50% sea water in both *Glycymeris* and *Mytilus*. Moreover, the part played by the amino acids in the isosmotic intracellular regulation of *Acanthochitona* and *Glycymeris* appears to be restricted. Indeed, in the sea water acclimated animals, the total amino-acid concentration accounts for about 2% of the total osmotic pressure in the muscle of *Acanthochitona* and 5% in *Glycymeris*. A greater osmoregulatory role appears to be played by taurine. This compound alone accounts indeed for about 2% of the total osmotic pressure in the muscle of *Acanthochitona*, 6% in *Glycymeris* and 3% in *Mytilus*. The important part played by taurine as a cellular osmotic effector appears to be of general occurrence in the phylum Mollusca (Awapara, 1962; Lange, 1963; Schoffeniels and Gilles, 1970a).

If amino acids and taurine can be considered as intracellular osmotic effectors, playing a part in the cellular osmotic regulation, nothing is known about the mechanisms implicated in the control of their concentration. Evidence has been given that, in Crustacea, the regulation of the intracellular amino-acid concentration, is due at least partly, to a mechanism involving modification of the cellular membrane permeability to amino acids (Gilles and Schoffeniels, 1969; Vincent-Marique and Gilles, 1970a, 1970b; Gerard and Gilles, 1971; Bouguegniau and Gilles, in preparation) and to a mechanism controlling, at the enzymatic level, the metabolism of the amino acids (Gilles, 1969; Schoffeniels and Gilles, 1970b). That modifications of the cellular membrane permeability are involved in the regulation of the amino-acid concentration in molluscs is indicated by the fact that upon exposure of isolated ventricles of *Modiolus* to a hypoosmotic stress, there is an increase in the efflux of the ninhydrin positive substances from the tissue into the saline (Pierce and Greenberg, 1970). On the other hand, the fact that in many euryhaline molluscs, osmotic acclimatization is paralleled by a modification of the oxygen consumption (Hiscock, 1953; Bielawski, 1961; Negus, 1968) and of the ammonia excretion (Emerson, 1969) can be interpreted as an indication of an increased degradation of the amino acids (Emerson, 1969). This is in agreement with the hypothesis according to which the regulation of the intracellular amino-acid pool may partly depend on a mechanism controlling the relative rate of anabolism and catabolism of these compounds (Gilles and Schoffeniels, 1964; Schoeffeniels, 1968; Gilles, 1969; Gilles and Schoffeniels, 1969). From the results obtained so far in this field with molluscan species, it is, however, hazardous to draw definitive conclusions. A study of the permeability to amino acids as well as of the amino-acid metabolism in tissues of euryhaline molluscs subjected to an osmotic stress is still lacking and further experimental results are needed to bring more evidence in favor of the interpretation given above.

SUMMARY

The molluscs *Glycymeris glycymeris*, *Mytilus edulis* and *Acanthochitona discrepans* can be acclimated from sea water to salinities down to 25% sea water. During this acclimatization, these molluscs do not show any extracellular anisosmotic regulatory power except for potassium, the blood level of which is regulated at the concentration it has in the blood of sea water aclimated animals.

During rapid acclimatization to diluted media, a transistory hyperosmotic state can be recorded in both blood and perivisceral fluid of the two bivalves species (*Glycymeris glycymeris* and *Mytilus edulis*) but not in the blood of the polyplacophora *Acanthochitona discrepans*. This hyperosmotic state, which can last for about 96 hours in both bivalves when placed suddenly in diluted media, is due to the ability of those molluscs to isolate themselves from the external medium by closing their valves tightly. This "shell-closing" mechanism may help the animals in withstanding a sudden osmotic stress but it cannot contribute to the osmotic regulation observed in these species.

In the studied species, it is the cell which has to cope with the osmotic stress and amino acids play a part in the cellular osmoregulation process. Taurine also appears as an important osmotic effector. Our results show that the concentration of the intracellular free amino acids and of taurine is higher in the muscle of sea water acclimated animals than in the same tissue taken from animals adapted to 50% sea water. The observed changes are greater than those which could be expected from the increase in the tissue water content recorded during the hypoosmotic stress.

The possible mechanisms implicated in the regulation of the amino acids and taurine concentration during the acclimatization of molluscs to diluted media are discussed.

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REGIONAL SURVEY OF GENE FREQUENCIES IN THE MUD SNAIL *NASSARIUS OBSOLETUS*

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One of the most abundant species of the littoral and estuarine environments of the Atlantic coast of the United States is the mud snail, *Nassarius obsoletus.* Adult snails are deposit feeders and scavengers occurring in large numbers on organic-rich intertidal flats from the Gulf of St. Lawrence in Canada to northern Florida (Scheltema, 1964). *Nassarius obsoletus* is an active species with a keen olfactory sense (Carr, 1967; Schaefer, 1969) and a tendency toward aggregative and schooling behavior (Jenner, 1956, 1957, 1958, 1959; Crisp, 1969). Dispersal is primarily by means of a planktonic larva (Scheltema, 1961, 1962).

Recently techniques combining zone electrophoresis and staining for specific proteins that are distinguishable as the products of individual gene loci have begun to contribute to the understanding of genetic systems of marine invertebrates (Manwell and Baker, 1970; Milkman and Beaty, 1970; Selander, Yang, Lewontin, and Johnson, 1970; Gooch and Schopf, 1970, 1971; Schopf and Gooch, 1971).

Electrophoresis and chromatography have had prior application in the genus *Nassarius.* Interspecific differences occur in protein patterns of chromatograms of British *Nassarius* (Collyer, 1961). Isozyme patterns of malate dehydrogenase in adult snails were studied by Meizel and Markert (1967), and electrophoretic activities of several enzymes in embryos and larvae were reported by Goldberg and Cather (1963) and Morrill and Norris (1965).

We report here the results of a study of the electrophoresis genetics of *N. obsoletus* which was begun with a twofold goal: (1) to discover and characterize gene loci from the pattern of their protein products on gels, and (2) to survey populations along a long coastal transect for geographic variation in the sampled portion of the genome.

Materials and Methods

Eleven collections of *N. obsoletus,* of 30 to 184 snails each, were made from January, 1969 to August, 1970 along a 1000 km Atlantic coast transect (Fig. 1). The transect ranges from Cape Cod Bay, Massachusetts, to near Beaufort, North Carolina, and embraces part or all of 3 zoogeographic provinces, the Acadian (1 collection), the Virginian (8), and the Carolinian (2).

Sampled populations represent a diversity of habitats: the sandy sublittoral (Cape Henlopen, Delaware), a salt marsh tidal creek (Canary Creek Marsh,

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Delaware), an estuary (Atlantic City, New Jersey), and intermittently exposed sand and mud flats (Barnstable Harbor and Eel Pond, Massachusetts; Southport, Old Mill Beach, and Stamford, Connecticut; Slaughter Beach, Delaware; and Pivers Island and Shell Point near Beaufort, North Carolina).

Snails were taken randomly by size and sex at most localities. Sites collected by the authors (all but Barnstable Harbor, Eel Pond, and Atlantic City) were sampled by taking all snails in the upper 2–3 cm of sediment of an area 1 m^2. Individuals were maintained alive and without food for a week or more to empty the digestive tract, since ingested matter may cause gel artifacts. They were then electrophoresed immediately or frozen at −60° C for later electrophoresis.

For each electrophoretic run snails were measured for apex to aperture height (Scheltema, 1964) and, when possible, were sexed (penis = male, nidamental gland = female). Sexing of some individuals proved very difficult because the copulatory apparatus of non-breeding snails is often inconspicuous (Jenner and Chamberlain, 1955), and genetic comparisons by sex are not presented below.

From each snail approximately 0.1 g of foot muscle was macerated with a ground glass rod in a 1 ml polystyrene centrifuge tube (Thomas Co., Philadelphia) in 100 μl cold 0.1 M Tris-glycine buffer, pH 8.5, and 25 per cent sucrose solution. Other tissues gave the same qualitative results when prepared identically. Tubes were centrifuged 20 minutes at 7000 rpm. Aliquots of 15 μl of supernatant were pipetted into slots in 6 per cent polyacrylamide gel (apparatus of E. C. Corp., Philadelphia) and gels were run vertically at 75–125 mA, 400–450 v at about 5° C for 2½ hours. Following electrophoresis gels were stained and bands were drawn or photographed for future reference.

Malate dehydrogenase (MDH) and lactate dehydrogenase (LDH)

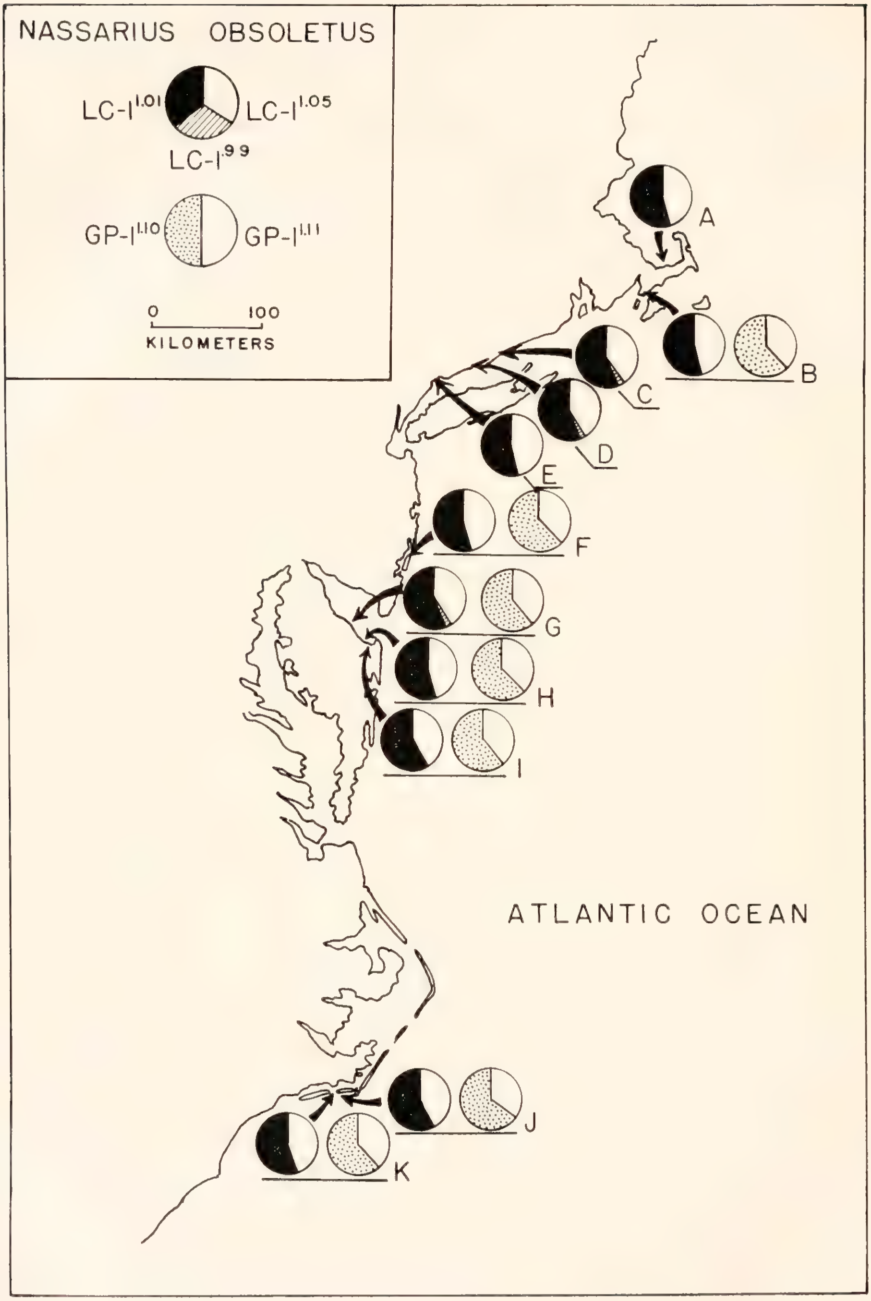
Electrophoresis buffer was Tris-glycine, pH 8.5, as above. Gels were stained in 0.04 M Tris-HCl buffer, pH 7.2, with a staining mixture of 30 mg NAD, 40 mg nitro BT chloride, and 3 mg phenazine methosulfate per 100 ml of solution. Substrates were the sodium salts of malic and lactic acid (Sigma Co., St. Louis). Bands of NAD-dependent MDH developed within 2 hours in the dark at room temperature, but the much lower assay of LDH required overnight staining.

General protein

Gel proteins were fixed in an aqueous solution of 12 per cent trichloroacetic acid and then stained 6–8 hours in a solution of 12 per cent trichloroacetic acid with 6 ml 1 per cent aqueous Coomassie Blue (Colab Co., Chicago) added per 100 ml.

Tetrazolium oxidase

Achromatic zones, operationally termed tetrazolium oxidase (Baur and Schorr, 1969), were visualized with the MDH and LDH staining systems with substrate omitted and staining in full light for 2–3 hours.


NASSARIUS OBSOLETUS
LC-I1.01
LC-I1.05
LC-I.99
GP-I1.10
GP-I1.11
0 100
KILOMETERS
A
B
C
D
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G
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I
J
K
ATLANTIC OCEAN

FIGURE 1. The transect of *Nassarius obsoletus* from Cape Cod, Massachusetts, to Beaufort, North Carolina. Allele frequencies at the Lc-1 locus are indicated as percentages of the shoreward circles and those of the Gp-1 locus by the seaward circles. Localities are:

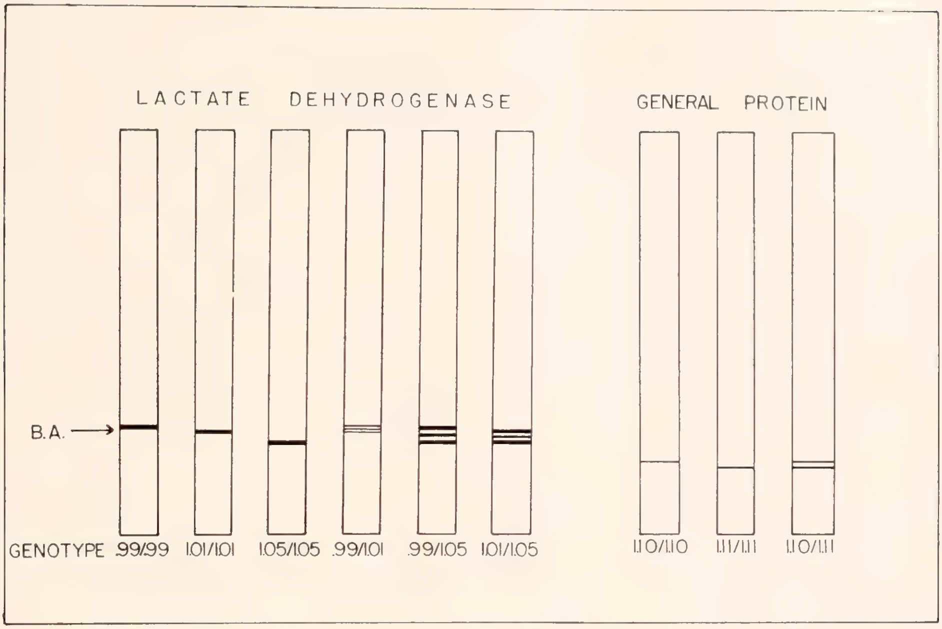

FIGURE 2. Diagram indicating band patterns and mobility relationships of protein zones belonging to the Lc-1 and Gp-1 loci. Homozygotes are single-banded and heterozygotes are three-banded (Lc-1 locus) and two-banded (Gp-1 locus). Arrow marks the mobility of bovine serum albumin under standard electrophoresis conditions.

Leucine aminopeptidase (LAP)

Electrophoresis buffer was 0.09 M Tris-borate, pH 8.8. Staining procedure was identical to that of Gooch and Schopf (1970) for LAP in ectoprocts.

Bovine serum albumin (Nutritional Biochemicals Corp.) was electrophoresed on each gel and stained with Coomassie Blue to serve as a reference standard for band mobility.

Other enzyme systems are under analysis. Esterase, glucose-6-phosphate dehydrogenase, and phosphohexose isomerase loci have been defined, but not studied along the transect. No activity was detected for ribonuclease, acid phosphatase, alpha-glycerophosphate dehydrogenase, and isocitrate dehydrogenase.

RESULTS

Gene loci

Six gene loci, 4 monomorphic (67 per cent) and 2 polymorphic (33 per cent), were characterized from gel patterns of the 5 protein systems (Table I). As with

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A, Barnstable Harbor, Massachusetts; B, Eel Pond, Massachusetts; C, Southport, Connecticut; D, Old Mill Beach, Connecticut; E, Stamford, Connecticut; F, Atlantic City, New Jersey; G, Slaughter Beach, Delaware; H, Canary Creek, Delaware; I, Cape Henlopen, Delaware; J, Shell Point, North Carolina; K, Pivers Island, North Carolina.

other organisms difficult or impossible to cross under controlled conditions, the interpretation of genotypes is based on electrophoresis patterns. See Selander, Yang, Lewontin, and Johnson (1970) and Gooch and Schopf (1970) for the detailed rationale. In brief, monomorphism is indicated for a locus where all individuals yield bands of identical mobility, and polymorphism is indicated where bands of 2 or more mobility classes occur, both singly and in all heterozygous combinations (Fig. 2).

Malate dehydrogenase. NAD-dependent malate dehydrogenase is the product of the locus M-1 with a single allele, M-1$^{.29}$, present in all populations (Table I). Superscripts of alleles refer to mobility of bands against the bovine albumin standard; nomenclature is discussed by Gooch and Schopf (1970). Under other electrophoretic conditions MDH may appear as 3 or more isozyme bands of variable mobility (Goldberg and Cather, 1963; Meizel and Markert, 1967), but the number of isozymes is apparently not genetic (Meizel and Markert, 1967).

TABLE I

Enzyme systems and defined gene loci in N. obsoletus

Enzyme system	Locus	Number and designation of alleles
General protein	Gp-1	2 Gp-1$^{1.10}$, Gp-1$^{1.11}$
Malate dehydrogenase	M-1	1 M-1$^{.29}$
Lactate dehydrogenase	Lc-1	3 Lc-1$^{.99}$, Lc-1$^{1.01}$, Lc-1$^{1.05}$
Tetrazolium oxidase	To-1	1 To-1$^{.44}$
	To-2	1 To-2$^{.71}$
"Leucine" aminopeptidase	Lap-1	1 Lap-1$^{.70}$

About 1 to 2 per cent of individuals of most populations show a broad, poorly defined band of MDH activity of the usual mobility. We cannot exclude the possibility of the diffuse zone representing a genetic variant. It will be treated operationally as identical to the allele characterized by the sharply defined band.

Lactate dehydrogenase. LDH is the product of the autosomal locus, Lc-1, with 3 codominant alleles, Lc-1$^{.99}$, Lc-1$^{1.01}$, and Lc-1$^{1.05}$ (Table I, Fig. 2). The presence of a hybrid band of intermediate mobility in heterozygotes denotes that LDH polypeptides probably associate as dimers.

General protein. Up to 8 faint and poorly defined band systems occur on gels stained for general protein. Protein bands of the locus Gp-1 are sharply resolved and permit the identification of 2 apparently autosomal and codominant alleles, Gp-1$^{1.10}$ and Gp-1$^{1.11}$ (Table I, Fig. 2). Heterozygotes are double-banded, indicating that the protein is probably not multimeric.

Tetrazolium oxidase. Two band systems occur on photocatalyzed tetrazolium-stained gels. They are operationally regarded as representing separate loci, To-1 and To-2. Both are monomorphic for single alleles, respectively, To-1$^{.44}$ and To-2$^{.71}$ (Table I). Three widely spaced populations only were surveyed for tetrazolium oxidase systems, Southport, Slaughter Beach, and Shell Point.

"Leucine" aminopeptidase. Gels stained for LAP possess a single zone of enzyme activity as the product of the locus Lap-1 (Table I). The Barnstable Harbor population was not surveyed for LAP.

Genetics of populations

The loci M-1, To-1, To-2, and Lap-1 are monomorphic for the same alleles in all sampled populations and thus do not contribute to regional genetic variation. The diallelic Gp-1 and triallelic Lc-1 loci segregate for the same alleles along the entire transect. The effects of age structure and geographic distance on allele and genotype frequencies were investigated.

Allele and genotype frequency in relation to age structure. The apex to aperture height increases with age in *N. obsoletus*, although individuals of a given age class may vary several mm (Morrison and Medcof, 1943; Scheltema, 1964). In some populations it is possible to distinguish age classes up to 2 years plus from discrete modal classes in height-frequency plots (Scheltema, 1964).

TABLE II

Snails from Connecticut populations partitioned into size classes. Genetic data for the Lc-1 locus and comparisons of genotype distributions with Hardy-Weinberg values are given for each size class

Size class (mm)	Sample size	Allele frequency		Genotype distribution			Chi-square accord with Hardy-Weinberg equilibrium
		Lc-$1^{1.01}$	Lc-$1^{1.05}$	1.01/1.01	1.01/1.05	1.05/1.05	
8–9	32	0.578	0.422	9	19	4	1.5, $p > 0.20$
10–11	65	0.523	0.477	16	36	13	0.8, $p > 0.30$
12–13	78	0.545	0.455	24	37	17	0.1, $p > 0.70$
14–15	95	0.584	0.416	34	43	18	0.4, $p > 0.50$
16–17	68	0.566	0.434	20	37	11	0.9, $p > 0.30$
18–21	30	0.600	0.400	11	14	5	0.02, $p > 0.80$
Pooled	368	0.561	0.439	114	186	68	0.3, $p > 0.50$

If natural selection has strong differential action on age classes, heterogeneity might appear in allele and genotype frequencies of populations of pooled size classes. A 2 × 6 table test of homogeneity for allele frequency *versus* size class at the Lc-1 locus for 368 snails of pooled Conneticut populations gives no evidence of heterogeneity (Table II; $\chi^2_{(5)} = 1.8$, $P > 0.8$; the rare genotypes bearing the 0.99 allele were not analyzed here or in subsequent comparisons). For feasibility of pooling procedure see next section. Homogenity chi-square for conformance of genotype distributions of size classes to Hardy-Weinberg expectations and pooled conformance also evidence non-significant values (homogeneity $\chi^2_{(5)} = 3.4$, $P > 0.6$; pooled $\chi^2_{(1)} = 0.4$, $P > 0.5$).

Tests of homogeneity of allele frequency with size class were also done for 136 snails from pooled Delaware populations. Nonhomogeneous distributions were not found for either locus (Lc-1 $\chi^2_{(3)} = 1.6$, $P > 0.5$; Gp-1 $\chi^2_{(3)} = 0.9$, $P > 0.8$).

Allele and genotype frequencies along the transect: Lc-1 Locus. Analysis of the Lc-1 locus of 746 individuals discloses the segregation of the 1.01 and 1.05 alleles in all populations of the transect (Table III). The 0.99 allele appeared solely in heterozygotes and in only 3 populations (Table III). Presumably more intensive sampling would reveal its presence in other populations. Overall allele frequencies are Lc-$1^{.99}$, 0.003; Lc-$1^{1.01}$, 0.548; and Lc-$1^{1.05}$, 0.449.

Genotype distributions in all populations agree with Hardy-Weinberg values (Table III). Pooled genotype distribution falls within Hardy-Weinberg limits ($\chi^2_{(1)} = 1.0$, $P > 0.3$; homogeneity $\chi^2_{(9)} = 16.1$, $P > .05$). Allele frequencies are also remarkably uniform (2×11 table homogeneity $\chi^2_{(10)} = 5.5$, $P > 0.8$). Even small inter-population differences in allele frequency should depress the pooled observed frequency of heterozygotes compared with Hardy-Weinberg frequencies based on pooled allele frequencies (Wahlund principle of Wallace, 1968). On the contrary an excess of 3 per cent observed hetrozygotes (non-significant) was found.

Table III

Allele frequencies and genotype distributions for polymorphic loci along the transect. Caption of Figure 1 gives the localities listed here by letters. Genetype distributions are compared to Hardy-Weinberg expectations by chi-square tests

Locality	Sample size	Allele frequency			Genotype distribution						Chi-square accord with Hardy-Weinberg equilibrium
Lc-q locus		Lc-$1^{0.99}$	Lc-$1^{1.01}$	Lc-$1^{1.05}$	0.99/ 0.99	0.99/ 1.01	0.99/ 1.05	1.01/ 1.01	1.01/ 1.05	1.05/ 1.05	
A	44	0	0.523	0.477	0	0	0	9	28	7	3.3, $P > 0.05$
B	54	0	0.528	0.472	0	0	0	12	33	9	2.8, $P > 0.05$
C	185	0.003	0.595	0.402	0	1	0	63	93	28	0.4, $P > 0.40$
D	129	0.004	0.531	0.465	0	0	1	37	63	28	0.02, $P > 0.80$
E	58	0	0.517	0.483	0	0	0	14	32	12	0.6, $P > 0.40$
F	30	0	0.517	0.483	0	0	0	10	11	9	2.1, $P > 0.10$
G	77	0.013	0.532	0.455	0	1	1	25	31	19	2.1, $P > 0.10$
H	31	0	0.516	0.484	0	0	0	10	12	9	1.5, $P > 0.20$
I	31	0	0.565	0.435	0	0	0	10	15	6	0.02, $P > 0.80$
J	54	0	0.574	0.426	0	0	0	16	30	8	1.0, $P > 0.30$
K	53	0	0.519	0.481	0	0	0	11	33	9	3.1, $P > 0.05$
Pooled	746	0.003	0.548	0.449	0	2	2	217	381	144	1.0, $P > 0.30$
Gp-1 locus		Gp-$1^{1.10}$	Gp-$1^{1.11}$			1.10/1.10		1.10/1.11	1.11/1.11		
B	52	0.615	0.385			20		24	8		0.02, $P > 0.80$
F	13	0.692	0.308			5		8	0*		2.0, $P > 0.10$
G	71	0.634	0.366			29		32	10		0.1, $P > 0.70$
H	32	0.656	0.344			12		18	2*		1.6, $P > 0.20$
I	32	0.688	0.312			14		16	2*		0.7, $P > 0.30$
J	47	0.713	0.287			21		25	1*		2.8, $P > 0.05$
K	48	0.635	0.354			21		19	8		1.0, $P > 0.30$
Pooled	295	0.654	0.346			122		142	31		0.1, $P > 0.70$

* Homozygote classes pooled.

Allele and genotype frequencies along the transect: Gp-1 Locus. Homogeneity of allele and genotype frequency is also manifest at the Gp-1 locus. In all 295 snails from 7 populations were surveyed. None of the genotype distributions differ significantly from Hardy-Weinberg values (Table III). Pooled genotypes fit Hardy-Weinberg values ($\chi^2_{(1)} = 0.1$, $P > 0.7$; homogeneity $\chi^2_{(5)} = 8.2$, $P > 0.1$). Again there is a non-significant excess of pooled observed heterozygotes, 6 per cent. Allele frequencies appear homogeneous (2×7 table, $\chi^2_{(6)} = 2.9$, $P > 0.8$).

Interaction between loci. Lacking knowledge of the linkage or epistatic relationships of the Lc-1 and Gp-1 loci, it cannot be presumed that their alleles assort independently or maintain random combinations. Linkage disequilibrium or positive or negative epistatic interactions may affect frequences of allelic combinations Deficiencies in certain gene combinations occurred in two "nothing" dehydrogenase loci in the polychaete *Hyalinoecia tubicola* (Manwell and Baker, 1970).

Genotypes at both loci were determined for 131 individuals from pooled Delaware populations (Table IV). Combination frequencies conform to random expectations ($\chi^2_{(7)} = 7.1$, $P > 0.3$), indicating the absence of detectable interaction between loci.

TABLE IV

Genotype combinations of the Lc-1 and Gp-1 loci in pooled Delaware populations. Figures in parentheses are values expected by random combination

Genotypes			
Gp-1 locus	Lc-1 Locus		
	1.01/1.01	1.01/1.05	1.05/1.05
1.10/1.10	21 (16.2)	23 (27.5)	11 (11.8)
1.10/1.11	15 (17.4)	28 (29.5)	18 (12.6)
1.11/1.11	7 (4.7)	5 (8.0)*	3 (3.4)*

* Pooled for chi-square.

DISCUSSION

The portion of the genome of *N. obsoletus* sampled by electrophoresis genetics presents a uniform picture along the coastal transect. At 4 loci single alleles have become established in all tested populations. At 2 polymorphic loci allele frequencies are virtually constant over the transect. Allele frequencies are homogeneous over age classes. Deviations from Hardy-Weinberg genotype distributions were not detected in 18 tests of individual populations and 2 of pooled populations.

Is the rest of the genome uniform geographically? Adaptive genetic differences along the transect should be manifested by geographically variable developmental and physiological tolerances. Clinal differences in developmental rate relative to temperature occur commonly in Pacific coast gastropods (Dehnel, 1955). There is no evidence for such differences in *N. obsoletus*. Temperature affects the rate of egg development of snails from Cape Cod and Beaufort almost identically (Scheltema, 1967). Reproductive timing in Atlantic Coast populations can be explained as response of the same genotype to environmental induction (Sastry, 1971). Adults from the cold waters of the Cape Cod Canal are remarkably tolerant to elevated water temperatures compared to other species of canal invertebrates examined (populations maintained at 35° C for 42 days before the onset of mortality: Pearce, Silverman, and LeGoff, 1968). The possession of higher thermal tolerance than is locally required is consistent with the hypothesis that the thermal adaptation, presumably genetic, of more southerly populations also exists in the Cape Cod Canal population.

The evidence presented above suggests that minimal genetic differentiation of populations exists along the transect and perhaps throughout the remainder of the species range. Exploring this hypothesis further, we may ask how such genetic uniformity is maintained. Regional uniformity of allele frequencies may be ascribed to (1) mechanisms of balancing selection, such as heterosis and frequency-dependent selection, or (2) pervasive gene flow opposing local selection and promoting regional panmixia.

Various kinds of balancing mechanisms have been proposed to explain the unexpectedly low variability in allele and karyotype frequencies found in some studies of animals with weak or moderate powers of dispersal (Carson, 1965; Prakash, Lewontin, and Hubby, 1969; Berger, 1971, using species of *Drosophila;* Burns and Johnson, 1971, on the butterfly *Hemiargus isole;* Koehn, Perez, and Merritt, 1971, in conjunction with the freshwater fish *Notropis stramineus*). In animals with greater dispersal abilities gene flow is usually held as the cause of regional stabilization of gene frequencies (Merrell, 1970, in reference to the *burnsi* gene in *Rana pipiens*).

Balancing mechanisms cannot maintain areal uniformity of allele frequencies if the adaptive values of genotypes are dependent on geographically variable elements of the environment. The more heterogeneous the environment the less likelihood there is of balancing selection alone maintaining uniformity. There is considerable environmental hetrogeneity along the coastal transect. The approximate yearly temperature variation at localities along the transect amounts to 0° to 18° C at the entrance of the Cape Cod Canal into Cape Cod Bay (Fairbanks, Collings, and Sides, 1968), 5° to 22° C off Cape Henlopen, Delaware (Cronin, Daiber, and Hulbert, 1962), and 7.9° to 27.6° C in the Beaufort estuaries (Williams, 1967). Temperature gradation along the transect is not entirely clinal. Sharp thermal discontinuities persist throughout much of the year off Cape Cod and Cape Hatteras and delimit the boundaries of zoogeographic provinces (Johnson, 1934; Hedgpeth, 1953; Cerame-Vivas and Gray, 1966). The winter thermal gradient near Cape Hatteras is sometimes a 14° C change over 8 nautical miles (Stefansson, Atkinson, and Bumpus, 1971). Inshore temperature variation is, of course, less drastic. These temperature differences exceed the tolerances of many species, limiting the species range or giving rise to physiological races (Segal, 1964; Vernberg and Vernberg, 1970).

Other aspects of the environment varied between collecting localities, but less systematically. Snails were collected on substrates varying from dark organic muds to coarse sands, and in salinities between 16‰ and 30‰. Undoubtedly variation occurred in current energy, diet, biotic association and other factors, but these were not studied in sufficient depth to evaluate here.

The remarkable physiological toughness of *N. obsoletus* that enables it to flourish in a diversity of environments may be partly due to heterotic mechanisms. However, we find it difficult to conceive of selection coefficients totally unresponsive to the environmental variation encountered by snail populations. For this reason balancing selection alone is not a convincing explanation for regional uniformity of allele frequencies.

Adult snails are capable of limited migratory activity (Batchelder, 1915; Crisp, 1969), but the true disperal stage and agent of gene flow is the planktonic larva. The efficacy of larval dispersal in widely distributed marine animals is well known (Johnson, 1939; Thorson, 1961; Robertson, 1964; Gurjanova, 1968;

Scheltema, 1966, 1968, 1971). Long-lived larvae may be transported transoceanic distances (Scheltema, 1971). In the shallow seas adjacent to continents tidal, rip, seasonal, and permanent currents distribute larvae over extensive areas (Mileikovsky, 1966). Off the Atlantic coast between Cape Cod and the Carolinas surface current drift is predominantly longshore with occasional reversals in direction, and velocities exceeding 10 miles per day are not uncommon (Bumpus, 1969).

In the laboratory the veliger of *N. obsoletus* persists a minimum of 10 days at 25° C and 21 days at 17.5° C (Scheltema, 1967), after which it may settle to the bottom. If conditions are unsuitable the veliger is followed by a pre-adult "creeping-swimming" stage which can postpone settlement many days in cold water (Scheltema, 1965) or where the substrate is unsuitable (Scheltema, 1961). Experimentally the total larval period may reach at least 53 days if sediment is withheld (Scheltema, personal communication). A larva remaining 53 days in the water column with a modest net longshore movement of 5 miles per day will travel 265 miles (423 km) before settling. Taking these figures conservatively as maximum estimates of dispersal per generation, a larva has the capability of travelling along 2/5 of the transect before it metamorphoses.

We infer from these figures that each local population is recruited from larvae of widely mixed provenance, and that extensive gene flow is largely responsible for the prevention of the formation of purely locally adapted gene complexes. Whether gene flow is sufficient to suppress all local genetic differentiation and to maintain complete regional panmixia is problematical and cannot be answered with the data at hand.

Greater insight may be provided if mechanisms of balancing selection and gene flow are viewed in concert rather than in isolation. Extensive gene flow would promote the selection of genes that interact harmoniously with many kinds of genetic background (Mayr, 1963), *i.e.*, they are "good mixers." The partial homogeneity of genetic background conferred by the spread of such genes would favor the diffusion of the same balancing mechanisms throughout the species. These mechanisms would not confront strictly locally adapted gene complexes in which they might have no adaptive value. Once established, balancing mechanisms would further dampen the centripetal tendencies of local adaptation.

Viewed in this way, gene flow and balanced polymorphisms may interact to retard or prevent the genetic "balkanization" of species. The natural history of *N. obsoletus* suggests that for this species gene flow has played the greater role.

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SUMMARY

Six gene loci were characterized by polyacrylamide gel electrophoresis of 5 protein systems in the mud snail, *Nassarius obsoletus*. Snail populations collected at 11 sites along an Atlantic coast transect from Cape Cod, Massachusetts to Beaufort, North Carolina were surveyed for genetic variability at these loci.

Four loci from malate dehydrogenase, tetrazolium oxidase, and leucine aminopeptidase systems are monomorphic for the same alleles througout the transect. Two loci from lactate dehydrogenase (Lc-1) and general protein ((Gp-1) systems are polymorphic in all populations.

The Lc-1 locus has 2 common and 1 rare codominant alleles and the Gp-1 locus segregates for 2 codominant alleles. Alleles of each locus segregate independently of those of the other. Chi-square homogeneity tests indicate homogeneity of allele and genotype frequency throughout the transect at both loci. All populations conform to Hardy-Weinberg equilibrium values, as do pooled populations without indication of Wahlund's effect. Populations partitioned into size (and hence age) classes are also homogeneous in allele frequency.

The remarkable geographic homogeneity of allele frequency may be due to (1) mechanisms of balanced polymorphism that are insensitive to local variations in environment, or (2) extensive gene flow. The potentialities of widespread pelagic dispersal in *N. obsoletus* argue strongly for gene flow; however the 2 agents of gene stablization are mutually complementary and may have acted in concert throughout populations.

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CAPTURE-RECAPTURE METHODS WITH *UCA*[1]

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A persistent problem in estimating animal population size is the choice of sampling and statistical methods appropriate to the behavior of the species under study. While censuses and controlled areal sampling estimates are suitable for sessile species, capture-mark-recapture methods may be used with mobile species. The maximum-likelihood index usually attributed to Lincoln (1930) and its well-known extensions by Jackson (1937, 1939) take the ratio of the marked and released animals to the total population to be equal to that of recaptured marked animals to the whole of the second capture. They share the assumptions that the marked animals immediately and randomly disperse into the population, that marked animals do not lose their marks, and that both marked and unmarked individuals are equally likely to be captured, marking and handling having no effect on viability or behavior. While the Lincoln Index requires a closed population, Jackson's two models allow for changes in population size during the period of sampling and provide for their specification as to deaths, births, emigration or immigration. Sampling differs in that the Lincoln Index is based on but one capture-mark-recapture sequence while Jackson's "positive" model employs a single marking followed by a series of recaptures and his "negative" method, a series of markings and a single, final recapture.

Casual observations on the behavior of sand fiddler crabs, *Uca pugilator* (Bosc) led us to the speculation that marked individuals might randomly disperse among the unmarked rapidly enough to permit an accurate estimate of population size by the Lincoln Index.

The sand fiddler as a test animal

The behavior of *Uca pugilator* is characterized by a cycle integrated with the stages of the tide. It is divisible into an active and an inactive phase. The active phase, on warm, clear days, occurs on the ebb tide and is made manifest by emergence from the burrows and promenading on the flats. During the inactive phase, which is triggered by the advance of the flood, the crabs retreat to burrows which, when the tide is high, may be covered with sand.

Uca pugilator is adapted to a different substrate than that of *U. rapax* (Smith), (Miller, 1961), the mud fiddler of Florida west coast, and can be easily distinguished from it in the field (Tashian and Vernberg, 1958). The sand fiddler is found in intertidal sandy shores sufficiently protected from wave action as to lack the properties of beaches. These often grade laterally into or may be

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surrounded by mud flats, and may be bounded on the landward side by salt marshes. These transitions form natural boundaries which ensure the discreteness of the sand fiddler populations.

Crane (1943, 1958) has described the tidal activity cycle of *Uca* species in detail. As the tide retreats, the burrows are cleaned and repaired; desultory ambulation, feeding, and mobbing follow. Near low tide, the mobs promenade, a group activity which may include feeding or may be preliminary to sexual display, courtship, and mating. Individuals may not participate in all phases of activity on any given tide, but all enter burrows on the flood and only a few refrain from joining the promenading mob to engage in other activities.

The mobbing phase of the sand fiddler's activity cycle seems, as we have noted, to be characterized by a randomness of movement which meets the primary assumption of the Lincoln Index, thereby overcoming the most usual stumbling block to its application. In the immobile burrowing phase, on the other hand, there seem to exist conditions suitable for population size estimates by controlled random areal sampling. It is the purpose of this paper to report the results of application of the Lincoln Index to a population of sand fiddlers, the results being compared with those obtained with the Jackson models applied also to the promenading mobs, and with sampling of the population during the immobile phase.

METHODS AND RESULTS

The study area

The seaward side of a sand spit lying between a salt marsh and an estuary in Wakulla County, Florida was chosen as the study area. The strand was 285 meters long at the high tide mark and varied in intertidal width from 4.1 to 7.2 meters. Its crest lay about 20 cm above the mean high water mark of spring tides. The tidal range was about 50–70 cm. The sediment consisted largely of medium fine sand with some shell hash and approximately 19% silt. The shoreward 2 or 3 meters of the intertidal were covered with *Spartina alterniflora* which was most densely distributed near the high water mark. The crest was loose sand covered with *Spartina patens* and the high water bush, *Iva frutescens*. At one end, the shore curved back into the *Juncus-Spartina* salt marsh along the high water line, but at low tide a northward projecting sand spit (Fig. 1 (6)) was laid bare. The habitat was chosen from many because of: (a) manageable size, (b) the presence of an apparently isolated population of sand fiddlers, and (c) accessibility.

Sampling

An initial plan to sample randomly during the mobile phase without subdividing the habitat was abandoned when it was noted that the population was already divided in two ways. First, a portion of the spit of about 315 m^2 was separated from the rest by an asphalt boat ramp which the fiddlers never cross (Fig. 1, A). Secondly, the population residing in the remainder was naturally subdivided into three mobs which regularly promenaded in clearly defined areas (Fig. 1, B, C, D). Color coded marking eventually showed that only three crabs were found outside their herding territories. This extremely contagious dispersion during the mobile phase called for restricted, or stratified, rather than simple random sampling.

Plastic pans (dimensions: 26 × 33 × 12 cm) were buried, rims flush with the sand surface, about two hours before ebb tide. Neither baiting nor driving was necessary; the normal activity of the mob caused some of the crabs to fall into the pans. The captured crabs were taken up at dead low water. Individual catches ranged between 185 and 567 crabs per pan. The carapaces were dried and marked with acrylic enamel, date and territory of capture being color coded.

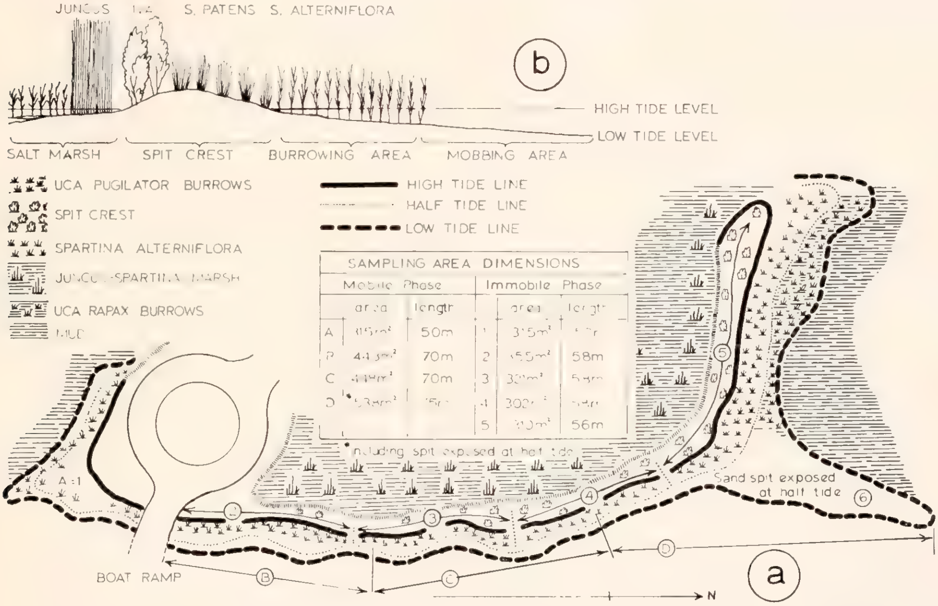
JUNCUS
S. PATENS
S. ALTERNIFLORA
b
HIGH TIDE LEVEL
LOW TIDE LEVEL
SALT MARSH
SPIT CREST
BURROWING AREA
MOBBING AREA
UCA PUGILATOR BURROWS
SPIT CREST
SPARTINA ALTERNIFLORA
JUNCUS-SPARTINA MARSH
UCA RAPAX BURROWS
MUD
HIGH TIDE LINE
HALF TIDE LINE
LOW TIDE LINE
SAMPLING AREA DIMENSIONS
Mobile Phase
Immobile Phase
area
length
A
50m
B
70m
C
70m
D
1
2
58m
3
4
5
56m
*including spit exposed at half tide
A
1
2
3
4
5
6
Sand spit exposed at half tide
BOAT RAMP
B
C
D
a
N

FIGURE 1. The study area. (a.) Plan of the sand spit shows areas sampled during the mobile phase (A, B, C, D) and areas sampled during the immobile phase (1, 2, 3, 4, 5) (not drawn to scale). (b.) Cross section of the sand spit shows the distribution of vegetation and the relationship between tide levels and activity areas of *Uca pugilator* (not drawn to scale).

The marks stood up best when placed near the posterior margin and could be seen clearly from a distance of five meters after three months. No modification of behavior was noted in marked crabs either immediately after release or during subsequent mobile activity phases. Immediate dispersal of the marked among unmarked members of the herd was observed in every case. Processing the catches always left ample time for dispersal into the mob before return to the burrows was triggered by the rising tide.

Samples were taken during the mobile phase on seven consecutive days—October 2 through 8, 1970—at the same relative time of the second or daylight tidal cycle. Data for the Lincoln Index were drawn from captures of those marked the previous day, while continuing mark-recapture data over the entire period were used in applying the Jackson models. In the analysis, the samples for all areas were combined in order to estimate the size of the whole population.

For random sampling during the immobile phase, the burrowing area was divided into five strata (Fig. 1, (1–5)), the boundaries of which were not all coincident with the apparent boundaries of the herding territories. Within each stratum, 1 m^2 quadrats were taken at random during the first half of the falling tide. Most quadrats were dug out to a depth of no new captures, usually about 50 cm or the low-tide level. Sample size was based on plotting cumulative density against quadrat number. When the curves levelled off, signifying that further sampling would yield no additional information, sampling was stopped. These density data were dealt with collectively for the entire sand spit.

Table I

Captures and recaptures of U. pugilator, *October 2 through 8, 1970*

Date and No. marked		Date recaptured						
		Oct. 3	Oct. 4	Oct. 5	Oct. 6	Oct. 7	Oct. 8	Total
Oct. 2	513	13	12	8	18	13	11	75
Oct. 3	1688		30	38	60	41	38	207
Oct. 4	1079			22	34	27	23	106
Oct. 5	1570				46	40	40	126
Oct. 6	1800					42	40	82
Oct. 7	1459						32	32
Oct. 8	1371							
Total recaptures		13	42	68	158	163	184	628
Total captures								8967

Results

Any mark-recapture data pair found in Table I may be used to estimate population size by the Lincoln Index. Taking, for example, the data from 10/2 and 10/3, we have

$$P = \frac{M \times C}{R} = \frac{513 \times 1688}{13} = 66611$$

The succeeding five data pairs yielded: 10/3–10/4: 60711; 10/4–10/5: 77001; 10/5–10/6: 61435; 10/6–10/7: 62529; 10/7–10/8: 62509.

In Table II, the data "corrected" to what they would be if 100 individuals had been captured and marked on each successive day are presented together with products computed to extrapolate back to the start of the experiment. The reader is referred to Jackson's papers (1937, 1939) or to Andrewartha (1961) for the computational methods.

TABLE II

Values for y, r, a_0 and P_0 for Jackson's positive and negative models (after Andrewartha, 1961)

Date marked and released	Date recaptured						r+	a_0	P pos.
	10/3	10/4	10/5	10/6	10/7	10/8			
10/2	0.150	0.216	0.099	0.195	0.173	0.156	0.988	0.183	54644
	10/3	0.165	0.143	0.198	0.166	0.164	1.023	0.150	66666**
		10/4	0.129	0.175	0.171	0.155	1.053	0.147	68027
			10/5	0.162	0.174	0.186	1.071	0.152	65789
				10/6	0.159	0.168	1.018	0.156	64102*
	r −	1.301	0.890	1.061	1.020	1.000			
	a_0	0.126	0.177	0.167	0.153	0.160			
	P neg.	79365*	56497	59880	65359	62500**			

* Values obtained using $r = y_n/y_{(n-1)}$.
** The two most reliable estimates.

From 85 one-square-meter quadrat sample units containing a total of 3472 crabs taken from burrows in an area of 1603 m^2, the population density was estimated to be 40.85 crabs/m^2 and the population size, 65483 individuals.

DISCUSSION

An indicator of the relative precision of the methods used here is the theoretical standard deviation of estimates. For the controlled sampling estimate this may be obtained by scaling up the variance of the sampling units and yields a result on the order of 10^3. Using maximum-likelihood techniques, Bailey (1951, 1952) gives the theoretical variance of the population estimate for the Lincoln method as

$$\text{var}\ \hat{x} = \frac{a^2 n (n - r)}{r^3}$$

in which a = the marked individuals, n = sample size, r = the number of recaptures, and $\hat{x}$ = the estimate of population size. This yields a standard deviation on the order of 10^4. For Jackson's negative method,

$$\text{var } \hat{x} = \left(\frac{\hat{x}}{n}\right)^2 \left[\left(\frac{\hat{x}\Sigma j^2 a_{(j)}}{(\Sigma j^2 a_{(j)})(\Sigma a_{(j)}) - \Sigma j a_{(j)}}\right) - 1\right]$$

for the j^{th} day where the death rate $= 0$. In this study, the standard deviation for Jackson's negative method is also on the order of 10^4. This may be assumed likewise for the positive method using the same data.

The population estimates yielded by application of the three capture-recapture methods to the mobile phase and that obtained by sampling the immobile phase lie close together. Since the variances of estimates of the population by the various methods are not homogeneous, testing the estimates on the assumption that they arise from the same population seems inappropriate. The following observations may be made, however.

(1.) The assumptions underlying the capture-recapture methods seem to be met in the mobile phase of *Uca pugilator.*
(2.) Despite the inherently greater variances of the capture-recapture methods, the estimates derived by their use may be more acceptable or preferred to controlled areal sampling estimates on the basis of time and energy investment, as well as of contributing little to disruption of the habitat or of the crabs' behavior.
(3.) The advantage of Jackson's methods over the Lincoln Index lies almost wholly in their ability to allow for changing population size and not on their having greater validity or precision of estimates.

The variances of the capture-recapture estimates are felt to be influenced by several recognizable sources of error. Part of it may be due to variation in environmental properties, such as the amount of cloud cover, the set and velocity of the wind, and the height of the sun as the tidal cycle advances through the daylight hours, which affect the willingness of the crabs to leave the burrows. Part of it is experimental, which is thought to include imprecision in placement of the traps relative both to the site of mobbing activity and to the stage of the tidal cycle, to influences of the presence of the traps upon behavior, to lack of knowledge of the true relationship of the mob to the area sampled during the immobile phase, and to the fact that one sub-population used a promenading area (Fig. 1, D) which was not contiguous to its burrowing area (Fig. 1, 5). A further source of error arises from the fact that the series of the estimates were based upon sub-populations of which varying proportions, from day to day, randomly remained outside the promenading mobs.

We recognize that an increase in the number of crabs captured would have served to reduce sampling error in two ways. First, larger captures would have represented a larger fraction of the population and would have allowed the estimates to be derived with less variance. Second, the importance of unusually large or small marking or recapture values in the Jackson models would have been minimized. In developing technique for sampling, these limitations were tolerated as preferable to the probable effects of: (1) the presence of several investigators in modifying the activity of the mobile phase over an increased time, (2) the effect of delaying collection of the trapped crabs until the rising tide altered the makeup of the promenading mob, and (3) the consequent return of marked crabs to a site different from that at which their behavior had caused their capture.

The Lincoln Index, in this study, was found to best estimate the population size when only successive day data were used. This agrees with the experience of Russell Hunter and Grant (1966) and is to be expected from the assumption of a closed population.

Andrewartha (1961) has pointed out that the examination of the y values of Jackson's methods will reveal indications of gains or losses in the population within the time span of the period of sampling. These indications may be verified by use of Jackson's formulae for gain or loss. No such trends were indicated in the y values computed during this study. This may be taken as further evidence of the appropriateness of the Lincoln Index as used here. No recruitment of mature fiddlers was expected or found during the mark-recapture period. The life cycle precludes this. The lack of clear evidence of mortality in an apparently high density crab population may indicate a scarcity of predatory birds.

The consistent identification of fiddlers with particular promenading mobs demands assumption of retention of this identity during the immobile stage. But, we have also been moved to wonder if the apparently continuously dispersed burrows can be divided either into clean cut segregations or, at least, into concentrations of fiddlers with particular promenading mob allegiances. Only general indications of the boundaries between such groups can be seen in the stratified sampling data, the random scatter of quadrats not having allowed for good linear coverage along the strand. This population of crabs belongs to Crane's (1943) shade-loving category, burrowing almost exclusively among the roots and stolons of *Spartina*. We have some indication that the zone of transition between the burrows of one sub-population and the next may occur where topography reduces or eliminates this cover. There follows, therefore, the inference that the mob is derived from a population occupying a circumscribable burrowing area.

The authors acknowledge with appreciation the contributions of Freda Hockett, who assisted with the field work, Katherine Kritzler, who typed the manuscript and Dennis S. Cassidy who assisted in preparation of the figure.

SUMMARY

1. Estimates of the size of a population of sand fiddler crabs, *Uca pugilator* (Bosc), were made with the Lincoln Index and Jackson's "positive" and "negative" models in the mobile phase of the activity cycle, and by stratified sampling of the immobile phase.

2. Restricted random sampling was employed because of extremely contagious distribution of the crabs during the mobile phase and of desire to achieve better coverage of the burrowing area than unrestricted random sampling might have yielded during the immobile phase.

3. During the mobile phase, the overall population was divided into four mobs, one separated from the rest by a physical, and the others from each other

by a behavioral barrier. Only three out of 8967 marked individuals were recaptured outside their home territories. Some evidence suggesting that the mobs are derived from subpopulations occupying circumscribable burrowing areas was obtained.

4. Appropriateness of the Lincoln Index was supported by immediate random dispersal of marked crabs among the unmarked mobs, and by failure of the Jackson methods to reveal evidence of population size change during the sampling period.

5. Recognition of the inhomogeneity of variances of estimates obtained by the several methods precluded statistical testing of the estimates on the assumption that they arise from the same population. While it is manifest that all the samples were drawn from the same over-all, isolated population of crabs, it is also obvious that the variance of one estimate was based on spatial distribution, while those of the others were based on behavior. Nevertheless, it was concluded that the assumptions implicit in capture-recapture methods are satisfied during the mobile activity phase of *Uca pugilator* and that for reasons of practicality and preservation of the habitat, these are preferable to estimates of population size by excisive, random, areal sampling during the immobile phase.

6. It is recognized that larger samples would have contributed to more satisfactory validation of the methods, to reduction of variance, and to positive bias. But it was concluded that those which formed the basis of this study were at least adequate, or even preferable, because of probable effects on the crabs' behavior of extended disturbance of mobbing and of the burrowing area.

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THE LARVAL DEVELOPMENT OF NORTHERN CALIFORNIA *PORCELLANIDAE* (DECAPODA, ANOMURA). I. *PACHYCHELES PUBESCENS* HOLMES IN COMPARISON TO *PACHYCHELES RUDIS* STIMPSON[1]

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The zoeae of the *Porcellanidae* (Decapoda, Anomura) possess extremely long rostral and posterior spines making them unique and easily identified in the plankton. However within the family, differences between genera and species are subtle and there is a growing interest in culturing the zoeae from hatching to determine taxonomically important characteristics. At present, larval descriptions for twenty species in seven genera are available. These are as follows:

- *Porcellana*
    - *P. platycheles* (Lebour, 1943; LeRoux, 1961)
    - *P. bluteli* (Bourdillon-Casanova, 1956)
    - *P. longicornis* (LeRoux, 1966)
    - *P. ornata* (Sankolli, 1967)
- *Polyonyx*
    - *P. quadriungulatus* (Knight, 1966)
    - *P. hendersoni* (Sankolli, 1967)
    - *P. gibbesi* (Gore, 1968)
- *Pisidia*
    - *P. longicornis* (Lebour, 1943)
    - *P. spinulifrons* (Sankolli, 1967)
- *Petrolisthes*
    - *P. armatus* (Lebour, 1943, 1950; Gore, 1970)
    - *P. rufescens* (Gohar and Al-Kholy, 1957)
    - *P. elongatus* (Greenwood, 1965)
    - *P. novaezelandiae* (Greenwood, 1965)
    - *P. lamarckii* (Sankolli, 1967)
- *Pachycheles*
    - *P. rudis* (Knight, 1966)
    - *P. haigae* (Boschi, Scelzo and Goldstein, 1967)
    - *P. natalensis* (Sankolli, 1967)
- *Euceramus*
    - *E. praelongus* (Roberts, 1968)
- *Megalobrachium*
    - *M. poeyi* (Gore, 1971)

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[1] Contribution Number 26 from the Pacific Marine Station, University of the Pacific, Dillon Beach, California 94929.

Two species of *Pachycheles, P. pubescens* Holmes and *P. rudis,* are found on the California coast north of San Francisco. The adults are distinguished on the basis of the shape of the carpus and carapace, by the hairiness of the chela, and by the number of telson plates on the female (Haig, 1960). Both species have been collected in rocky intertidal regions from British Columbia to Baja California.

As noted above, Knight (1966) cultured and described the zoeae of *P. rudis.* The present paper presents a description of the larval development of *P. pubescens.* Because of the similarity of the two species, *P. rudis* was cultured along with *P. pubescens. Pachycheles rudis* zoeae were found to be identical to Knight's (1966) description. A subsequent paper will contain larval descriptions of the genus *Petrolisthes,* which is the only other genus of *Porcellanidae* on the northern California coast, and it should then be possible to identify *Porcellanidae* zoeae collected from this area to the species level.

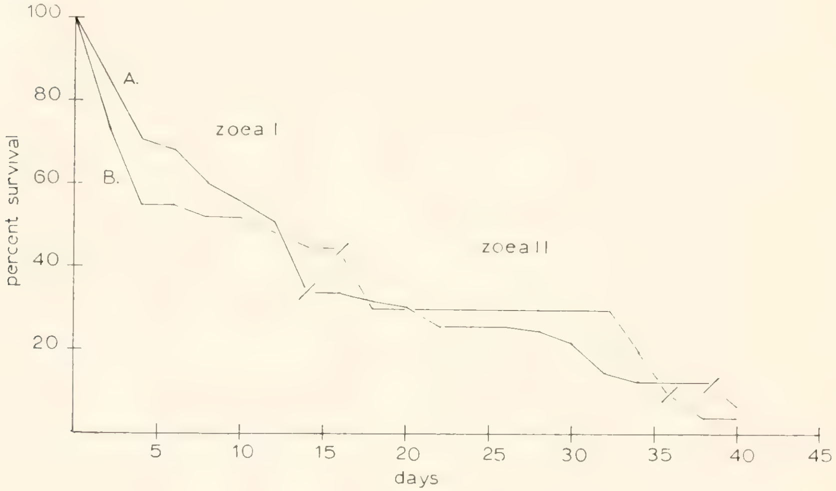

FIGURE 1. The per cent survival of *Pachycheles pubescens* Holmes plotted at two day intervals for the duration of its pelagic life. One hundred per cent represents 68 zoea in series A, 20 zoea in series B.

MATERIALS AND METHODS

The larvae of *P. pubescens* were found to be almost identical to those of *P. rudis* at hatching and it was necessary to culture series of zoea of both species simultaneously. To further substantiate the results, series of zoea were cultured from two females of each species.

Three ovigerous females of *Pachycheles pubescens* and two of *Pachycheles rudis* were collected during −1.0 tides on the north jetty, Doran Park, Sonoma County, California, in October, January and February, 1970–1971. The adults were maintained separately in plastic aquaria on sea water tables until the zoea hatched.

Zoeae were transferred to 62 mm stender dishes containing approximately 25 cc of filtered sea water. Two zoeae were cultured per dish. The dishes were covered and placed in a dark refrigerator at 14° C ± 1° C. Salinity ranged from 30.0‰ to 34.0‰. During the period of the experiment the temperature and salinity of the ocean were monitored. Ocean temperature ranged from 9 to 14° C; salinity ranged from 32.0 to 36.0‰.

TABLE I

Measurements (mm) of the carapace, rostrum, posterior spine and rostrum/posterior spine ratio in the zoea of Pachycheles pubescens *and* P. rudis

	carapace	rostrum	p. spine	ros./p. spine
Zoea I				
P. pubescens (ser. A)				
mean	1.48	5.18	2.52	2.10
range	1.38–1.63	4.85–5.63	2.25–2.75	1.87–2.25
no. examined	15	15	15	15
P. pubescens (ser. B)				
mean	1.56	5.42	2.50	2.19
range	1.50–1.63	4.63–5.75	2.38–2.63	1.82–2.70
no. examined	15	15	15	15
P. rudis				
mean	1.49	5.33	2.80	1.93
range	1.38–1.60	5.00–5.75	2.38–3.00	1.75–2.34
no. examined	20	19	19	18
P. rudis (Knight, 1966)				
mean	—	5.78	3.00	—
range	—	5.64–5.92	2.92–3.08	—
no. examined	—	15	17	—
Zoea II				
P. pubescens				
mean	2.28	6.92	2.84	2.45
range	2.18–2.38	6.13–7.75	2.50–3.40	2.13–2.64
no. examined	6	6	6	6
P. rudis (Knight, 1966)				
mean	—	8.39	3.69	—
range	—	8.00–9.08	3.48–3.88	—
no. examined	—	7	6	—

Every second day the zoeae were transferred with a large bore plastic pipette into dishes containing fresh filtered sea water at 14° C. At this time a drop of concentrated culture of newly hatched *Artemia salina* (L.) nauplii was added as food, and the number of deaths and molts were counted.

Measurements of the whole zoeae, accurate to 0.05 mm, were made under a stereo-microscope with an ocular micrometer. Carapace measurements were made from the base of the rostrum to the base of the posterior spines. Only intact rostra and posterior spines were measured. Specimens of each developmental stage and exuvia were preserved in ethylene glycol.

Propylene phenoxetol was used as a narcotizing agent. Specimens of each stage of each species were dissected and the appendages examined under bright field illumination on a Leitz OrthoLux compound microscope. Drawings and measurements of the appendages were made with the aid of a camera lucida.

RESULTS

Ovigerous females of *Pachycheles pubescens* were kept as long as 49 days before the eggs hatched. The females, with carapace widths from 10.0 to 12.5 mm, produced 25, 68 and 107 zoeae. The first zoeal stage lasted from 8 to 16 days and from 34 to 45 per cent survived to the second stage. The total pelagic larval phase extended from 34 to 40 days and there was only a 4 to 5 per cent survival to glaucothoe. In Figure 1, the per cent survival of the first and second zoeal stages is graphed for every 48 hour period. In both series there was a high mortality in the first zoeal stage, particularly during the first four days after hatching. A second high mortality occurred before the glaucothöe; in fact, a number of zoeae actually died in the process of molting to this stage. These two periods seem to be the most critical in the larval development. It is probable that there are critical temperature, salinity or other physiological requirements at this time (Costlow, Bookhout and Monroe, 1960).

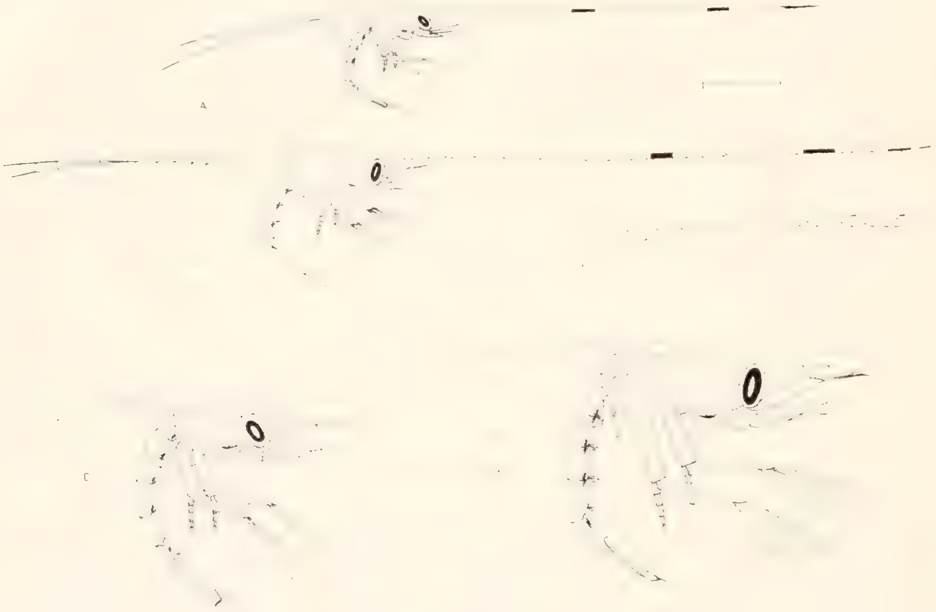

FIGURE 2. The zoeal stages of *Pachycheles pubescens* Holmes: A, D, first zoea; B, E, second zoea; C, setation of rostrum. Scale lines equal 1.0 mm.

Measurements of the carapace, rostrum, posterior spines and ratio of the rostral to the posterior spines for the two zoeal stages of *P. pubescens* and *P. rudis* are presented in Table I. There is no significant difference in size between the first zoeal stages of the two species. However, in Knight's (1966) culturing of *P. rudis,* the rostrum and posterior spines of the second zoeal stage were longer than those of *P. pubescens.* Knight also reported that larger specimens of *P. rudis* were collected in the plankton. It is probable that size is variable and in part determined by one or more environmental factors.

Description

The carapace, abdomen and appendages of each stage are described with respect to setation and, in some cases, to relative size. Plumose and non-plumose setae have not been differentiated in the text; the reader is asked to refer to the illustrations for these details.

Zoea I

Carapace (Fig. 2A). The carapace is shallow, inflated and produced into a long rostrum and two posterior spines. The rostrum bears spinules which are concentrated on the ventral surface. The spinules completely encircle the rostrum only in the mid region of the length (Fig. 2C). The posterior spines bear a single row of 14 or 15 ventral spines.

Antennule (Fig. 3A). The unsegmented antennule bears six terminal processes: 3 aesthetes, 2 plumose setae, and one hair.

Antenna (Fig. 3B). The endopodite is fused to the protopodite and has a small hair on the inner margin. The exopodite is a movable spine which is almost 2 times as long as the endopodite and nine times as long as it is wide. It is armed with 3, or occasionally 4, curved hooks on the inner margin. On the intact animal the antenna is slightly shorter than the antennule.

Mandibles (Fig. 3C). The mandibles are asymetrical with small teeth and molar processes. Palps are absent.

Maxillule (Fig. 3D). The endopodite is unsegmented and bears 3 terminal setae and a single strong subterminal seta. There is a row of setules on the outer border. The basal endite has teeth and three setae; the coxal endite has 9 long curved processes.

Maxilla (Fig. 3E). The scaphognathite bears 6 setae along the lateral margin and a single long seta on the apex. Setules are present between the setae. The endopodite has 6 terminal and 2 subterminal setae and setules along the outer border. The basal endite has 7–8 processes on the distal lobe and 6–7 on the proximal lobe. The coxal endite bears 4 processes on the distal lobe and 8 on the proximal lobe.

First maxilliped (Fig. 3F). Two setae are present on the inner margin of the coxopodite. The basipodite has 10 setae along the margin in groups of 2, 2, 3 and 3 progressing distally. The endopodite is four segmented. The distal segment bears 9 terminal setae and 1 long plumose subterminal seta. The third segment has 3 distal setae and 1 medial seta; the first and second segments each have 3 setae. There are no setules on the outer margin of the endopodite. The exopodite is indistinctly 2 segmented and ends with 4 natatory setae.

Second maxilliped (Fig. 3G). The coxopodite is unarmed. The basipodite has 3 setae in groups of 1 and 2 progressing distally. The endopodite is four

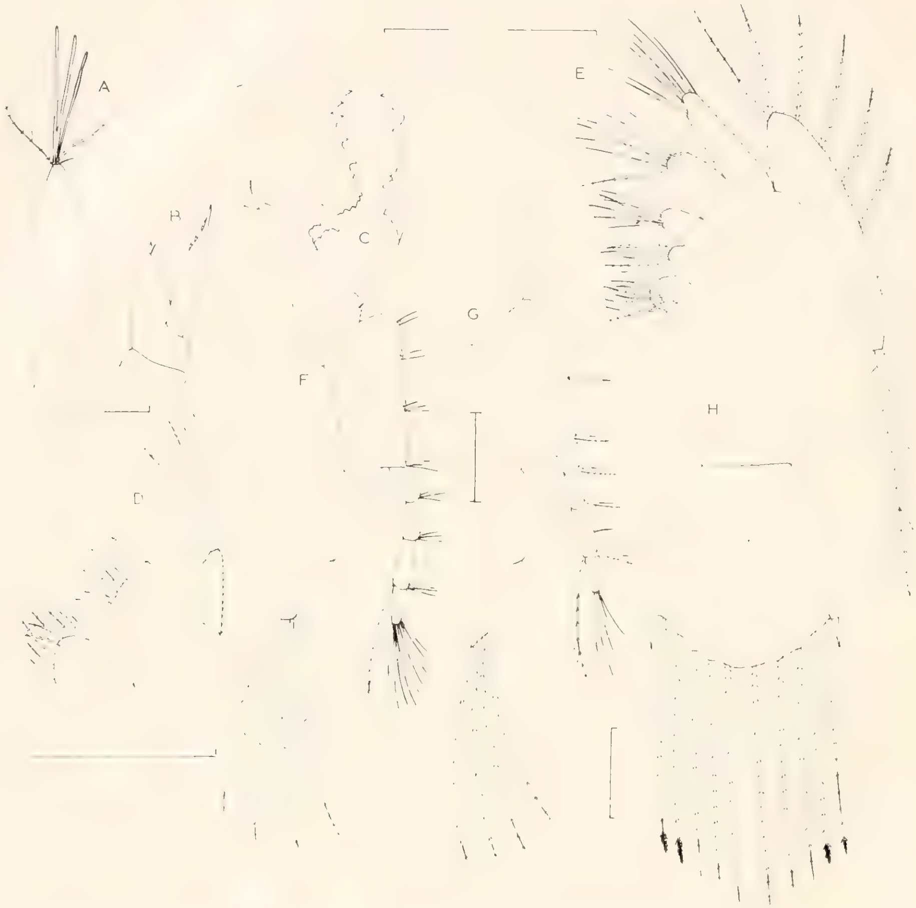

Figure 3. *Pachycheles pubescens* Holmes: first zoeal appendages; A, antennule; B, antenna; C, mandibles; D, maxillule; E, maxilla, F, first maxilliped; G, second maxilliped; H, third maxilliped; I, telson. Scale lines equal 0.2 mm.

segmented. The distal segment bears 5 terminal setae and 1 long subterminal seta. The third segment has 2 distal setae and 1 medial seta; the first and second segments each bear 2 setae. There are no setules on the inner margin of the endopodite. The exopodite is indistinctly 2 segmented and ends with four natatory setae.

Third maxilliped and periopods. The third maxilliped and periopods are rudimentary buds beneath the carapace.

Abdomen (Fig. 2D). The abdomen consists of five somites. Each somite has a row of fine teeth around the dorsal margin and somites 4 and 5 have small lateral spines.

Telson (Fig. 3H). The telson is as long as it is wide, and bears the standard $7 + 7$ terminal processes. The third and fourth processes (first and second long spines) are each armed with curved hooks at the tips. An anal spine is present.

Coloration (Fig. 2D). A pair of red chromatophores is present around the mouth, on the abdominal somites 1 through 4 and on the telson. An orange-red

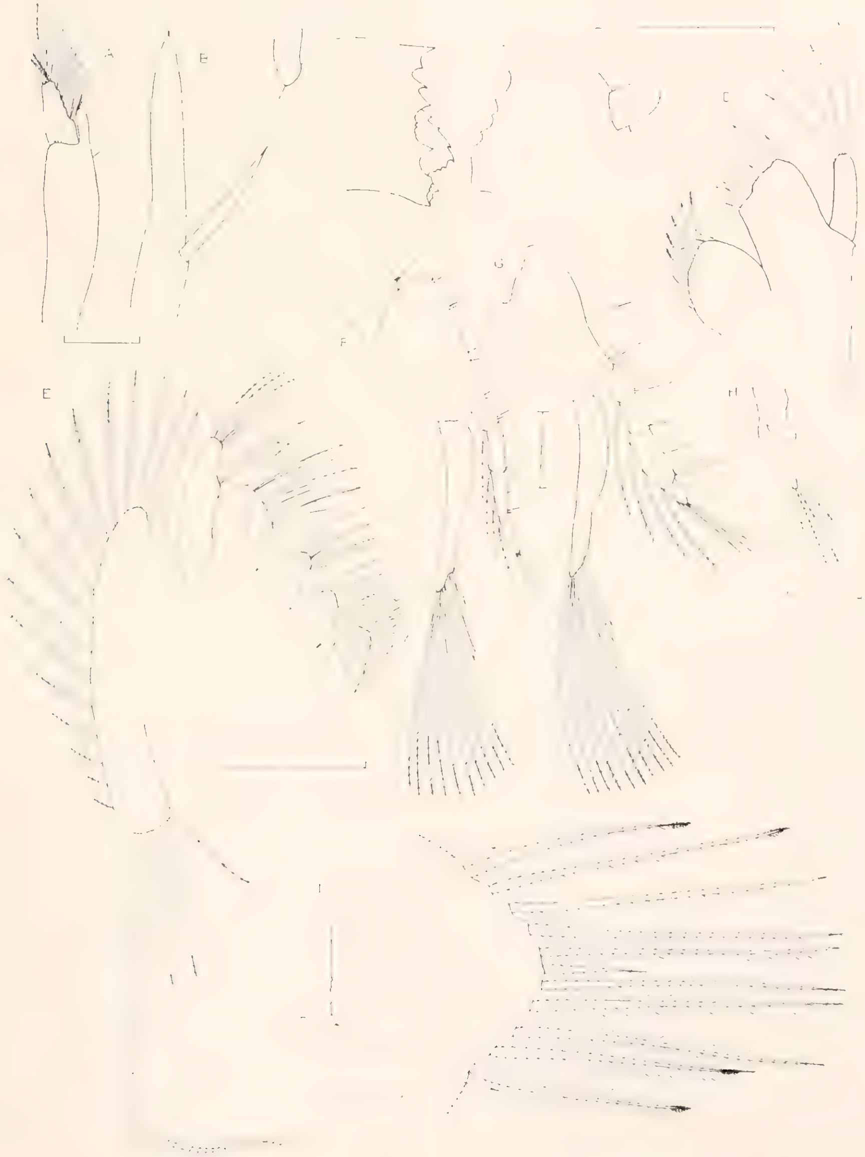

FIGURE 4. *Pachycheles pubescens* Holmes: second zoeal appendages; A, antennule; B, antenna; C, mandibles; D, maxillule; E, maxilla; F, first maxilliped; G, second maxilliped; H, third maxilliped; I, telson. Scale lines equal 0.2 mm.

coloration is apparent on the tips of the posterior spines and forms three bands around the rostrum.

Zoea II

The eyes are definitely stalked, the exopodites of the maxillipeds bear 12–14 natatory setae and pleopod buds are present on the abdominal somites.

Antennule (Fig. 4A). The antennule is biramous. The exopodite bears 7 terminal processes: 3 aesthetates, 1 long and 2 small plumose setae and a small hair. There are five tiers of aesthetates along the inner margin in groups of 4, 4, 2, 2 and 2 progressing distally. The endopodite is unarmed and fused to the protopodite. The protopodite bears a single seta on the distal corner.

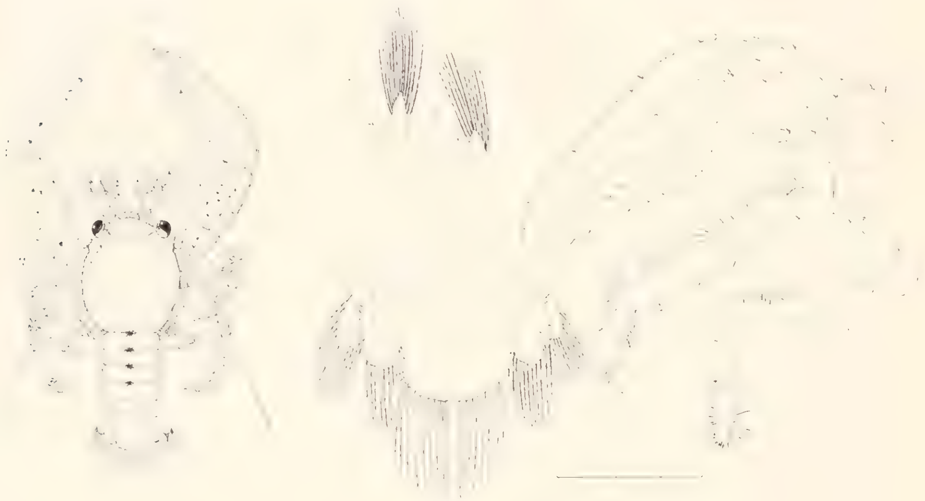

FIGURE 5. *Pachycheles pubescens* Holmes: Glaucothöe and glaucothöe locomotory appendages; A, first pleopod; B, fourth pleopod; C, telson; D, cheliped; E, walking leg; F, fifth periopod. Scale lines equal 0.5 mm.

Antenna (Fig. 4B). The endopodite has greatly increased in relative length and terminates in a small tooth and a hair. The exopodite has lost the hooks on the inner margin.

Mandibles (Fig. 4C). The mandibles now possess a rudimentary palp.

Maxillule (Fig. 4D). The endopodite is unchanged. The basal endite bears 5 long curved spines and 6 setae.

Maxilla (Fig. 4E). The scaphognathite has 16–18 setae around the lateral margin. In addition there are 4 apical setae of which the outermost is by far the longest. The endopodite has 6 terminal and 3 subterminal setae. The basal endite bears 10 processes on the distal lobe and 9–10 on the proximal lobe; the coxal endite has 6–7 on the distal lobe and 10–12 on the proximal lobe.

First maxilliped (Fig. 4F). The exopodite now terminates in 14 natatory setae. On the endopodite, segments 1 through 4 each have an additional long seta on the outer margin. The terminal segment ends in 10 setae.

Second maxilliped (Fig. 4G). The exopodite now terminates in 12 natatory setae. The first three segments of the endopodite have an additional long seta on the outer margin. The third segment is swollen.

Third maxilliped (Fig. 4H). The endopodite is rudimentary and consists of a swollen bud. The exopodite bears four terminal setae.

Periopods. The peripods have increased in size beneath the carapace.

Pleopods. Pleopod buds are present on abdominal somites 2, 3, 4 and 5.

Telson (Fig. 4I). The telson has an additional spine on the central prominence.

Glaucothöe

Carapace (Fig. 5). The carapace is sharply serrated on the anterior margin and dentate along the lateral margins. Length × width: 1.5 × 1.4 mm.

Antennule (Fig. 6A). The antennule is biramous. The peduncle is three segmented; the basal segment is enlarged and dentate. The outer ramus is five segmented and bears a row of long aesthetates on the inner border. The lower ramus is three segmented with a group of long setae on the distal margin of the proximal segment. Other setae are placed as illustrated.

Antenna (Fig. 6B). The peduncle is three segmented. The proximal segment has a blade-like extension on the outer margin. The flagellum consists of about 25 segments each with several short setae on the anterior margin.

Mandibles (Fig. 6C). The teeth have been reduced; the palp is three segmented with 10 short spines on the terminal segment.

Maxillule (Fig. 6D). The endopodite is unsegmented and bears 4 setae. The basal endite has about 15 teeth and 9 setae; the coxal endite has about 30 spines.

Maxilla (Fig. 6E). The scaphognathite is fringed with 51 setae. The endopodite is unsegmented and has 2 long setae. The basal endite is armed with about 27 processes on the distal lobe and about 16 on the proximal lobe. The distal lobe of the coxal endite bears about 10 terminal setae and 10 subterminal setae. The proximal lobe bears 15 subterminal and 19 terminal setae.

First maxilliped (Fig. 6F). The exopodite and endopodite are reduced and unsegmented. The exopodite has 6 setae along the outer margin. The protopodite bears about 40 processes on the basal lobe and 11 on the coxal lobe.

Second maxilliped (Fig. 6G). The exopodite is two segmented. The distal segment has 6 terminal and 4 subterminal setae. The proximal segment has 4 or 5 setae. The endopodite is four segmented. Progressing proximally these segments are armed with about 18, 15, 5 and 7 setae. The basipodite has 9 setae on the outer margin and 1 on the inner margin. The coxopodite has 6 setae on the outer margin.

Third maxilliped (Fig. 6H). The exopodite is unsegmented and devoid of setae. The endopodite consists of 5 segments. The distal segment bears 7 long setae. The outer borders of the next three segments each are lined with from 7 to 13 setae. The fourth segment from the tip is armed with a toothed blade-like extension. The most proximal segment has 9 setae on the outer border.

Periopods (Fig. 5D, E, F). The subequal chelipeds are serrate along the edges and sparsely covered with tubercles and setae. The carpus has two large teeth on the inner margin (Fig. 5D). The walking legs are covered with long setae which are most dense on the ventral margin. The dactyls have 3 spines

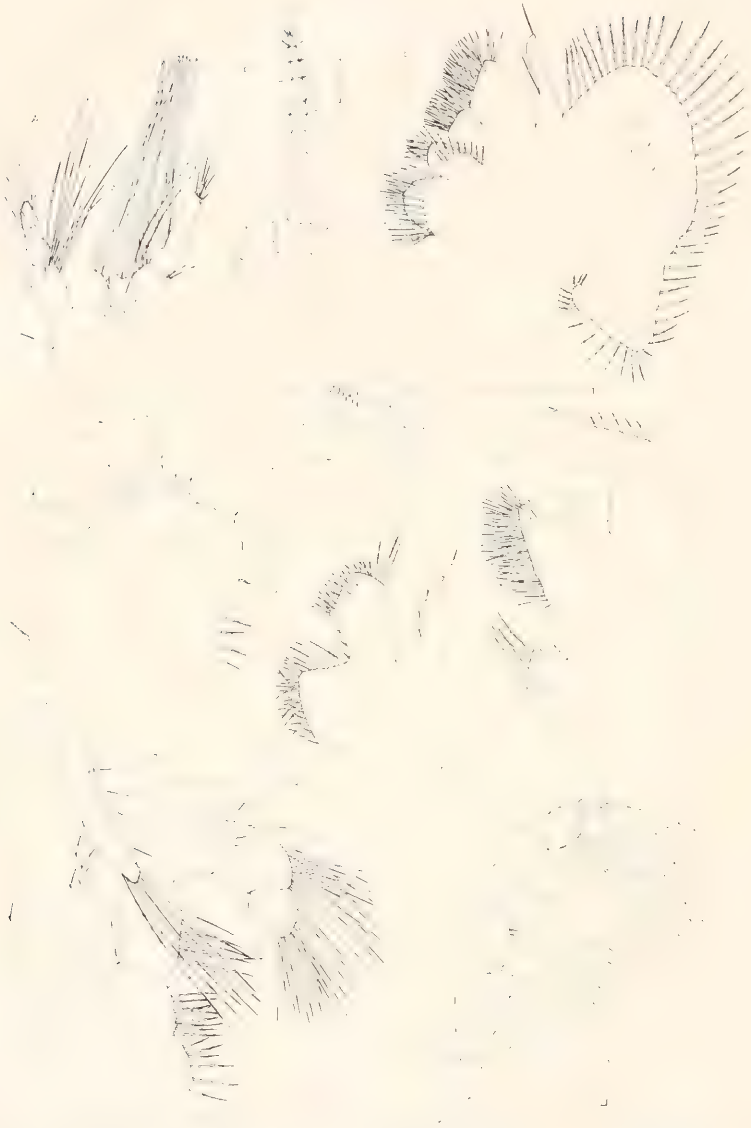

FIGURE 6. *Pachycheles pubescens* Holmes: Glaucothoe sensory and feeding appendages; A, antennule; B, antenna; C, mandible; D, maxillule; E, maxilla; F, first maxilliped; G, second maxilliped; H, third maxilliped. Scale lines equal 0.2 mm.

on the inner margin. The propodus has 2 spines on the anterior dorsal corner and 2 more on the dorsal margin (Fig. 5E). The fifth periopod is chelate. The chelae are armed with from 4 to 6 scythe-like hooks and numerous setae (Fig. 5F).

Pleopods (Fig. 5A, B). There are four pair of Pleopods which decrease in size from the second to the fifth somite. The setation is variable. The exopodites

TABLE II

Summary of distinguishing morphological characteristics of Pachycheles pubescens, P. rudis (*Knight, 1966*), P. haigae (*Boschi et al., 1967*) and P. natalensis (*Sankolli, 1967*)

	P. pubescens	*P. rudis*	*P. haigae*	*P. natalensis*
Zoea I				
Antenna				
exopodite, no. hooks	3–4	3	4	3
Maxillule				
basal endite, no. setae	9	9	9	8
Maxilla				
scaphognathite, no. setae	6	6	6	7
endopodite, no. setae	12	12	12	8–9
First maxilliped				
basipodite, no. setae	9	9	9	10
endopodite				
4th segment, no. setae	9–10	7–8	10	8
3rd segment, no. setae	3 + 1	3 + 2	4 + 3	6
2nd segment, no. setae	3	3	2	4
1st segment, no. setae	2	2	3	2
Second maxilliped				
endopodite				
4th segment, no. setae	5	5	7	5
3rd segment, no. setae	3	3	2	3
1st & 2nd segments, no. setae	2	2	3	2
Telson, no. spines with hooks	4	4	4	10
Zoea II				
Mandibles, palps	+	+	0	—
Maxilla				
scaphognathite, no. lateral setae	17–18	20–22	18	—
First maxilliped				
endopodite				
inner margin, no. setae	4	3	4	—
4th segment, no. setae	10	8	10–12	—
Second maxilliped				
endopodite, no. setae on inner margin	4	3	4	—
Third maxilliped, no. setae	4	2	2	—

have from 10 to 13 setae. The endopodites have from 2 to 4 small hooks and a single seta.

Telson (Fig. 5C). The telson is a semicircular plate with a distinct medial notch. The posterior border has 17 long setae and 14 shorter setae. Hooks characteristic of the zoeal telson setae are not present in the glaucothöe. The ventral surface is sparsely covered with short setae. The uropods have 14 setae on the exopodite and 11 setae on the endopodite. There are also 5 short setae on the outer border of the endopodite.

Coloration (Fig. 5). Red chromatophores are distributed on the carapace, eyestalks, periopods and mouth parts. Four large red chromatophores are present between the abdominal segments.

DISCUSSION

The zoeae of *P. pubescens* are very similar to those of *P. rudis*. The first zoeal stages can be distinguished only by the setation of the endopodite of the first maxillipeds. In *P. pubescens*, the fourth segment has 9 or 10 setae and the third segment has 1 medial seta. In *P. rudis* there are only 7 or 8 terminal setae on the fourth segment and 2 medial setae on the third (Table II). The species diverge in the second stage. In addition to the differences listed above, there are four long setae on the inner margin of the endopodite of the first and second maxillipeds in *P. pubescens* and only three in *P. rudis*. *P. pubescens* has four setae on the exopodite of the third maxilliped; *P. rudis* has only two. The scaphognathite of the maxilla also differs. In *P. pubescens* there are 17–18 setae along the lateral margins; in *P. rudis* there are 20–22.

The two California species have more in common with each other than they do with other described species. Because the zoeae of the two species are so similar, Knight's (1966) comparison of *P. rudis* zoeae collected from the plankton with those cultured in the laboratory is questionable.

Knight (1966) did not describe the appendages of the *P. rudis* glaucothöe. Only one specimen was obtained from the present culturing of that species which was in poor condition. It was evident however that the carpus, which is an important taxonomic characteristic in the adult, can be used to distinguish the glaucothöe of the two species. The anterior margin of the carpus is produced into two large spines in *P. pubescens* and only one in *P. rudis*.

The zoea of the northern California species differ from *P. haigae* (Boschi *et al.*, 1967) in the number of hooks on the antennal exopodite and in the setation of the second maxilliped in the first stage, and in the lack of palps on the mandibles in the second stage. In addition, the Indian species, *P. natalensis* (Sankolli, 1967), differs in the setation of the maxillule and maxilla and in the number of telson spines with hooks at the tips (Table II).

The chromatophore distribution is identical in *P. pubescens* and *P. natalensis* (Sankolli, 1967). The descriptions of *P. rudis* (Knight, 1966) and *P. haigae* (Boschi *et al.*, 1967) do not include notes on coloration. However *P. rudis* cultured at this laboratory was found to have the same pattern of chromatophore distribution as *P. pubescens* and *P. natalensis*. This color pattern may prove to be characteristic of the genus, but at this point too few species have been described to make any generalizations. Other characteristics which may prove peculiar to the genus, particularly in differentiating it from *Petrolisthes*, may be the lack of setules on the endopodites of the first and second maxillipeds in zoea I and the two outermost pair of telson spines only with hooks on the tips (although *P. natalensis* differs in the second respect).

In conclusion, *Pachycheles pubescens* and *Pachycheles rudis* are almost identical at hatching and diverge later in development. The two species seem to be more closely related to each other than they are to other described species. The first maxilliped is the most variable feature between species within the genus. Chromatophore distribution, number of telson spines tipped with hooks, and the absence of setules on the inner margin of the endopodites of the maxillipeds may prove to be important characteristics of the genus.

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SUMMARY

Two species of *Pachycheles* (Decapoda, Porcellanidae) occur along the northern California coast: *P. pubescens* and *P. rudis*. The zoeae of the two species are very similar. At hatching they differ only in the setation of the first maxilliped, however they diverge later in development. The setation of the first maxilliped appears to be the most variable feature of species of this genus so far described. Chromatophore distribution, number of telson spines with hooks at the tip, and the absence of setules on the inner margin of the endopodites of the maxillipeds may prove to be important in differentiating *Pachycheles* from other *Porcellanidae*, particularly from the genus *Petrolisthes*.

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THE EFFECTS OF OXYGEN CONCENTRATION AND ANOXIA ON RESPIRATION OF *ABARENICOLA PACIFICA* AND *LUMBRINERIS ZONATA* (POLYCHAETA)[1, 2]

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Animals respond in two general ways to a decrease in external oxygen concentration, *viz.*, they maintain the same respiratory rate independent of external oxygen concentrations down to a certain critical oxygen concentration (regulation) or their respiratory rate is directly dependent upon oxygen concentration (nonregulation) (Florey, 1966). Some examples of the effect of oxygen concentration on respiratory rate are reviewed by Beadle (1961).

When subjected to anoxic or nearly anoxic conditions, organisms will either undergo anaerobic metabolism, reduce their metabolic rate, or die. In the first case, partially oxidized end products can be accumulated in the organism, *i.e.*, the organism incurs an oxygen debt. Payment of the oxygen debt is indicated by an increased rate of oxygen uptake by the organism upon returning to aerobic conditions. Studies on the response of animals to anoxia have been reviewed by Brand (1944) and Beadle (1961).

The purpose of my studies was to measure oxygen concentrations in burrows of *Abarenicola pacifica* Healy and Wells at different stages of the tide; to investigate the effect of oxygen concentration on the respiratory rates of *A. pacifica* and *Lumbrineris zonata* (Johnson); and to determine if either species incurs an oxygen debt during anoxia. These worms live in large numbers at about +0.8 m above mean lower low water in muddy sand at False Bay, San Juan Island, Washington. Experiments were conducted during September 1968 to June 1969 at the Friday Harbor Laboratories, Friday Harbor, Washington.

The ecology of *A. pacifica* has been studied in False Bay by Hobson (1966, 1967). Lugworms irrigate their J-shaped burrows during high tide, drawing in overlying water, but not during low tide. Hobson (1966) demonstrated that *A. pacifica* performs aerial respiration, thus increasing its supply of oxygen at low tide. Wells (1945, 1949, 1953) has made extensive ecological observations on the related species, *Arenicola marina*. To my knowledge the only previous observations on *L. zonata* concern their geographic distribution (Hartman, 1944, 1948) and their rate of oxygen uptake in aerated water (Pamatmat, 1968; Banse,

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Nichols and May, 1971). Pamatmat (1968) has investigated physical and chemical parameters in False Bay and also the ecology and metabolism of the entire benthic community there.

METHODS

Oxygen concentration in burrows

Laboratory studies were made in a tank of running sea water. Specimens of *A. pacifica* were allowed to establish burrows in sand from their habitat in the tank. The tank was drained for subaerial exposure of the burrows and was filled to simulate subaqueous conditions in the field.

The volume of water in a 10-cm deep burrow with a 2-gram worm was estimated to be 4 ml. Samples of water were obtained from burrows in both the field and the laboratory by inserting a 9-cm long, 15-gauge, hypodermic needle into a burrow and slowly drawing about 1 ml of sample into a 5-ml glass syringe. The tip of the needle was covered with 35-μ mesh nylon netting to preclude entry of sediment. Water from burrows was clear. If turbid water was withdrawn, indicating possible contamination by interstitial water, the sample was discarded. The dead space in the needle and syringe was filled with water from the same or an adjacent burrow. After removal of any bubbles of air from the sample, the syringe was immediately sealed with a toothpick and was placed in ice until the time of analysis (1 to 4 hr later). The samples were usually kept in the dark to prevent photosynthesis by microalgae included in the sample. Sediment temperature was measured by inserting a laboratory thermometer into the sand to a depth of 8 cm, the usual depth of sampling for water from burrows. Lugworms were usually below this depth.

In the laboratory the partial pressure of oxygen of the samples was measured with a Physiological Gas Analyzer, Model 160 (Beckman Instruments, Inc.) using a constant temperature bath, or with a Blood Micro System BM3 with Acid-Base Analyzer PHM71 (Radiometer A/S, Copenhagen, Denmark). Barometric pressure was measured with a Precision Microbarograph. The analyzers were calibrated with gas mixtures that had been analyzed with a 0.5-cc analyzer (Scholander, 1947) for the percentage of oxygen. To convert tensions to oxygen concentrations, it was assumed that the salinity of water in the field was 31‰, on the basis of the measurements of interstitial and overlying water in False Bay by Pamatmat (1968). Salinity in the sea water tanks was 30.5‰ in April and 30.0‰ in May. The oxygen concentration of sea water of known temperature and salinity in equilibrium with air was obtained from a nomogram based upon data from Green (1965).

Tests were run to determine whether the samples obtained from burrows were being contaminated with interstitial water. A solution of Evans blue dye was injected into the sand at several points 1 to 3 cm from a burrow in the tank, after which a sample was drawn from the burrow and its absorbance was measured at 602 mμ. At the same time a sample was taken from a burrow about 0.5 m away from the dye injection point as a control. These tests indicated that samples from lugworm burrows were not contaminated with interstital water.

Weights

Respiratory rates were calculated using wet weights. So that these data can be compared with data from other sources, the relationships between wet weight, dry weight, and ash-free dry weight were determined for *A. pacifica.* Prior to these determinations, worms were kept in sea water for at least 1 day to reduce the contents of their guts by defecation. Wet weights of *A. pacifica* were measured after blotting live specimens for a few seconds with absorbent tissue paper. Because of the small size of *L. zonata,* it was necessary to blot the live specimens for 5 minutes to obtain reproducible wet weights. Dry weights of *A. pacifica* were determined after drying at 80° C for 3 days, by which time the weight was constant. To determine ash content of lugworms, specimens were burned in a muffle furnace. The temperature of the furnace was increased gradually from 200° C to 500–550° C during an 8-hr period and left at 500–550° C for 8 hr more.

Respiratory rates

Specimens were all collected within a few meters of each other at about the 0.8-m tide level (100 m SW of Station 1 of Pamatmat, 1968) and in sediments that appeared similar in texture and color. For measurements of respiratory rates, worms were removed from sand in either the field or the laboratory and were placed in sea water for about 1 day in order to decrease the contents of their guts. Filtered sea water (Gelman glass fiber filter) was aerated and then siphoned into glass-stoppered (BOD), 300-ml bottles. Each test was made in the dark and consisted of at least one control bottle containing filtered water only and one experimental bottle with one or two *A. pacifica* or five to seven *L. zonata,* which were rinsed in filtered water before being put into the bottles. The tests lasted 0.9 to 3.5 hr for *A. pacifica* and 2.0 to 2.5 hr for *L. zonata.* The change in oxygen concentration during a test was not more than about 1.3 ml O_2/l for *A. pacifica* or not more than 0.6 ml O_2/l for *L. zonata.* At the end of the test, water was siphoned from the bottles and analyzed for oxygen concentration by the Winkler method. A 50-ml subsample was titrated with approximately 0.0085 N $Na_2S_2O_3$, standardized with 0.0100 N $KH(IO_3)_2$. Wet weights were measured at the end of an experiment (a series of tests on the same worms).

To study the effect of oxygen concentration on respiratory rate, rates of the same worms were measured repeatedly, using different initial oxygen concentrations. These were obtained by bubbling nitrogen gas through the water. In each experiment the order of the various oxygen concentrations was selected more or less randomly. Each experiment lasted from 1 to 4 days for *A. pacifica* or for 1 day for *L. zonata.*

The relationship between wet weight and respiratory rate of *A. pacifica* was determined by a total of 34 measurements of rate using 28 worms, collected and tested in February. In the aerated water used for all tests, oxygen concentrations ranged from 5.73 to 6.82 ml O_2/l, which is somewhat below the saturation values.

Oxygen debt

Experiments were run to determine whether the worms incurred an oxygen debt during anoxia and repaid this debt upon returning to aerated water. Their respiratory rate was measured once or twice in aerated water to give initial rates; they were subjected to anoxia; then their respiratory rate was measured in aerated water immediately after they were removed from anoxia. Nitrogen gas was bubbled through sea water, resulting in an oxygen concentration of about 0.1 to 0.5 ml O_2/l. Worms were left in this water from 1 to 3 days. They can be assumed to have consumed the small amount of oxygen present within a short time after being placed in this low concentration and to have experienced anoxia for the remainder of the test. Consequently, the exact length of time in anoxia is not known. Measurements of oxygen concentration of the water in which the worms had been held resulted in values of 0.0 to 0.1 ml O_2/l. The Winkler method can be in error by 0.1 ml O_2/l at low-oxygen concentrations because of atmospheric contamination (Broenkow and Cline, 1969). In most cases a control set of worms in aerated water was run simultaneously with the experimental group to detect changes in respiratory rate due to causes other than oxygen deprivation, *e.g.*, starvation, absence of a burrow, or accumulation of metabolites in the enclosed water. The bottles of the control worms were kept unstoppered, and the water was aerated periodically by bubbling compressed air through the water.

TABLE I

Oxygen concentration in burrows of Abarenicola pacifica *in False Bay*

Date	Tide	N*	Oxygen concentration, ml O_2/l			Comments
			Mean	S.D.§	Range	
Apr 26	Low	6	0.76	0.20	0.66–1.16	On sand bar
Apr 26	Low	2	0.80		0.74–0.87	In tide pool
Apr 27	Low	5	0.79	0.35	0.49–1.35	One burrow only
Apr 27	Low	5	0.69	0.22	0.41–0.98	One burrow only
Apr 29	Low	5	1.17	0.45	0.60–1.79	On sand bar
Apr 29	Low	3	1.41	0.32	1.11–1.75	In tide pool
Apr 29	High	2	1.04		0.60–1.49	Early high tide
Apr 30	Low	4	0.90	0.39	0.46–1.35	On sand bar
Apr 30	High	2	1.88		1.25–2.50	Early high tide
Apr 30	High	4	2.49	1.10	1.39–3.93	Later high tide
May 1	High	8	2.08	1.02	1.50–4.35	Late high tide
May 21	High	7	2.93	1.28	1.28–5.31	Late high tide

* Number of burrows sampled (except where noted under Comments).
§ Standard deviation.

RESULTS

Oxygen concentration in burrows

The results of measurements of oxygen concentration in burrows of *A. pacifica* in False Bay at low tide indicate that water in these burrows is never anoxic (Table I). The lowest value was 0.41 ml O_2/l. The mean value from Table I for low tide was 0.9 ml O_2/l. Twelve preliminary measurements in burrows in the field at low tide on September 20 gave a mean of 1.1 ml O_2/l. The mean value from Table I for high tide was 2.3 ml O_2/l. The mean for early high tide was 1.5 ml O_2/l by comparison with a mean of 2.5 ml O_2/l for 19 measurements taken later in high tide. Because worms cannot irrigate their burrows until covered by water, they may not have completely flushed their burrows at the time the samples were taken during early high tide. The results of May 1 and May 21 suggests the highest values that might be expected, not only because the samples were taken near the end of high tide when the burrows probably were well irrigated, but also because the overlying water was observed to be supersaturated (6.53, 6.78 and 7.58 ml O_2/l or 132, 113 and 114 per cent saturation on May 21). Twelve measurements from three different burrows in the laboratory had a mean of 0.9 ml O_2/l for subaerial exposure, soon after the overlying water was drained off. Twelve similar measurements had a mean of 1.4 ml O_2/l for subaqueous conditions, which had been continuous for 1.5 days. The walls of the burrows in the field, though not always in the laboratory, are tan, indicating an oxidized lining in contrast with the dark gray reduced sediment beyond the burrows.

TABLE II

Respiratory relationships of Abarenicola pacifica

Equation No.	Regression coefficients*				R^2	F	df
	a	b	c	d			
5	0.54	1.01			0.62	160.3	1,99
6	0.93	0.86	−0.51		0.70	28.4	2,98
7	−0.36	0.86	−0.38	1.33	0.74	14.0	3,97
8	1.49		−0.22		0.55	39.8	1,32

*See equation (4).

All of the 200 or more *L. zonata* excavated during low tide from the sand were found in the layer of gray reduced sand below the 2-cm-thick surface tan layer. The burrow walls of some worms were tan, although other walls were gray. I never observed any of their burrows to be continuous to the surface in the field. None of their burrows connected with the aerated burrows of *A. pacifica* or *Upogebia* sp. found in the same vicinity. Because some of the lumbrinerid burrows have tan walls, these worms must contact either the surface of the sand or at least the oxidized surface layer at some time. These worms in mud in the laboratory will readily burrow to the surface of the sediment; however, they rapidly retreat from the surface when they are disturbed.

Weights

Wet, dry, and ash-free dry weights were determined on 10 specimens of *A. pacifica* that had been kept in sea water for 1 day and on 12 specimens that had been kept in sea water for 5 days. The mean percentage dry weight of wet weight for the 22 measurements was 14.1 per cent with a standard deviation of 1.3 per cent, while the mean percentage ash-free dry weight of wet weight was 11.0 per cent with a standard deviation of 1.3 per cent. The percentage ash-free dry weight of dry weight for the first set (75.6%) was significantly different from the value for the second set (79.5%) at the 1 per cent level ($t = 4.40 > t_{0.01} = 3.106$). On the basis of the percentages of ash-free dry weight of dry weight, it is probable that keeping the worms in sea water for longer than 1 day results in further defecation. The amount of additional defecation is so small, however, that it is almost inconsequential. Because many experiments lasted 3 or 4 days, a mean percentage for both sets of 77.7 per cent (standard deviation = 1.3%) ash-free dry weight of dry weight is probably most representative of the weight relationships of worms in these experiments.

Respiratory rate vs. oxygen concentration, weight, and temperature

Oxygen uptake rate can be expressed as a linear function of concentration for *A. pacifica.* Nineteen measurements of a representative 5.65-gram worm using a temperature range of 7.2 to 9.2° C and an oxygen concentration range of 0.43 to 6.45 ml O_2/l resulted in the following equation (Fig. 1):

$$R = 0.49 + (3.15)([O_2]) \tag{1}$$

where R is the oxygen uptake rate in microliters oxygen per gram wet weight per hour and $[O_2]$ is mean oxygen concentration for a test in milliliters oxygen per liter. The y-intercept is not significantly different from zero ($t = 0.155 < t_{0.50} = 0.689$) at the 50 per cent level. The correlation coefficient squared is 0.82, which shows that 82 per cent of the variability of respiration in this case could be explained by the variation of oxygen concentration. Another equation (Fig. 1)

$$R = 1.84 + (4.23)([O_2]) \tag{2}$$

resulted from 20 measurements on a 3.15-gram worm using a temperature range of 7.8 to 10.0° C and a range of oxygen concentration of 1.29 to 6.60 ml O_2/l. It is noteworthy that the slope is larger than in equation (1) because this worm was smaller. The correlation coefficient squared is 0.83. Both equations are significant at the 1 per cent level.

The relationship between respiratory rate and oxygen concentration, weight, and temperature is the following for *A. pacifica:*

$$R = 10^a [O_2]^b W^c T^d, \tag{3}$$

which also can be expressed in the following form:

$$\log R = a + b \log [O_2] + c \log W + d \log T. \tag{4}$$

On the basis of 101 measurements on *A. pacifica* from December 28 to March 23, a multiple regression of respiratory rate on oxygen concentration, weight, and temperature was computed. The regression has the form of equation (4) where W is wet weight in grams and T is temperature in degrees Celsius. Each step of the BIMED stepwise computation (Dixon, 1968) is shown in Table II as equations (5), (6) and (7), which are significant at the 1 per cent level. The ranges of the parameters are shown in Table III. Because the coefficient "b" in equation (5) is almost exactly one, oxygen consumption rate is in fact a linear function of oxygen concentration.

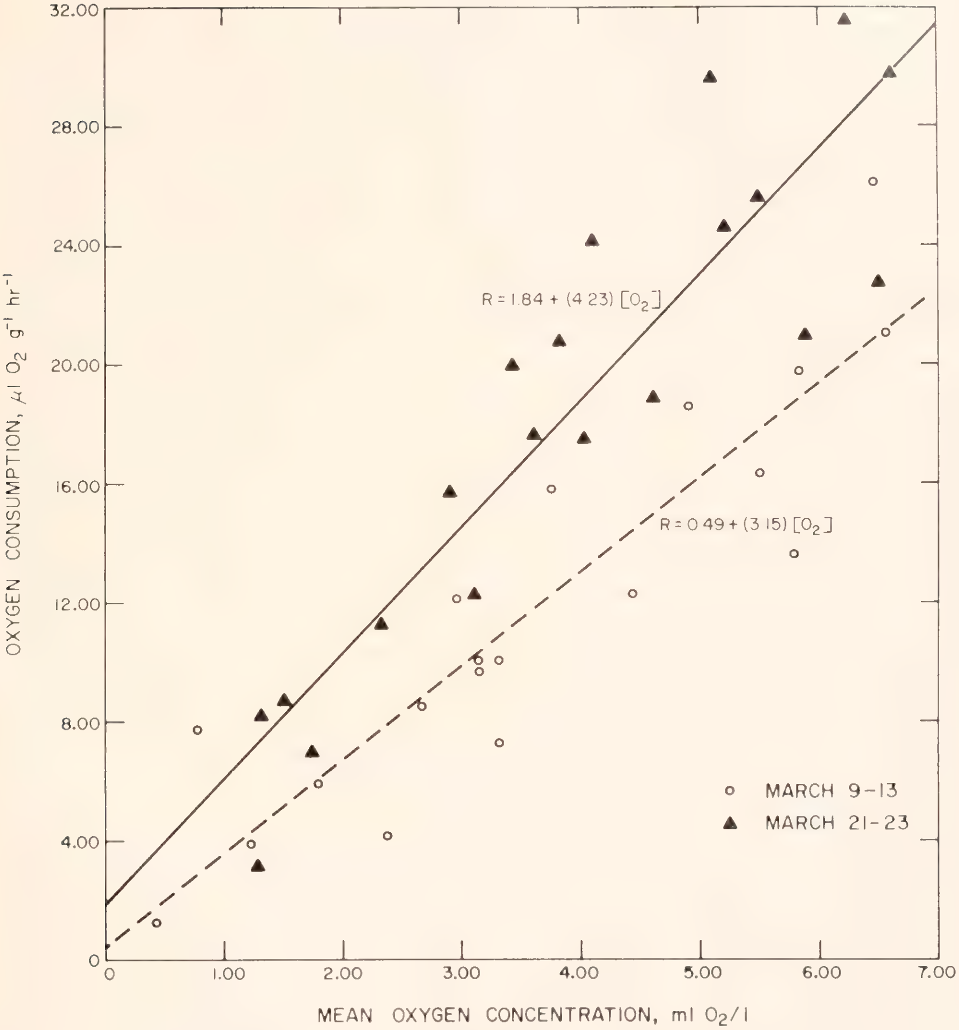

Figure 1. Oxygen consumption of *Abarenicola pacifica* as a function of oxygen concentration.

Equation (8) may also be expressed as

$$\log R' = 1.49 + 0.78 \log W \tag{9}$$

where R′ is in microliters oxygen per worm per hr. The correlation coefficient squared for this equation is 0.94.

Using equation (7) a mean daily respiratory rate can be calculated for the period of late December through March 1969. Assuming a 2-gram worm, a temperature of 8° C, and an oxygen concentration of 2.3 ml O_2/l, the mean respiratory rate would be 21.6 μl O_2 worm^{-1}hr^{-1} during high tide. For the same worm at low tide at 4° C and 0.9 ml O_2/l, the mean rate would be 3.9 μl O_2 worm^{-1}hr^{-1}.

Table III

Ranges of parameters and number of determinations for equations in Table II

Equation No.	$[O_2]$ range ml O_2/l	Weight range g	Temp. range C	N
5, 6, 7	0.43–6.71	1.18–7.52	6.1–10.0	101
8	5.73–6.82	0.20–6.33	7.1–8.4	34

Graphs of tidal height as a function of time were prepared from data in a tide table, assuming that tidal height is a sinusoidal function of time. These graphs indicate that organisms at the +0.8-m tidal level experienced an average of 6 hr of low tide and 18 hr of high tide per day during January through March 1969. Consequently, the mean respiratory rate would be 0.4 ml O_2 worm^{1-}day^{-1}.

In contrast with *A. pacifica*, *L. zonata* probably regulates its respiratory rate between 6 ml O_2/l and about 2 ml O_2/l, which is the critical oxygen concentration below which it is a nonregulator. Figure 2 shows the results for six sets of worms where the individuals had a range in mean wet weight of 0.16 to 0.21 g; the experiments were run in a range of temperature of 11.6 to 14.7° C and of oxygen concentration of 0.95 to 6.14 ml O_2/l. There were four to eight measurements on each set of worms.

Oxygen debt

Results of seven experiments to test the presence or absence of an oxygen debt in *A. pacifica* were equivocal and did not substantiate either the presence or absence of an oxygen debt.

Two experiments on *L. zonata* are shown in Table IV. With the exception of the second experimental in the second experiment, all experimentals have a substantially higher rate after anoxia than before. These preliminary experiments indicate that *L. zonata* contracts an oxygen debt during anoxia.

Table IV

Experiments on oxygen debt

Dates	Control or experimental	R, μl $O_2 g^{-1} hr^{-1}$			Duration of anoxia, hr
		Initial	Initial	Final	
Lumbrineris zonata					
Jun 5–7	Experimental	58.1	56.6	95.8	40
	Experimental	64.0	60.7	88.1	40
	Control	49.5	54.0	62.6	40
Jun 9–11	Experimental	48.2	55.4	82.5	50
	Experimental	68.3	74.5	78.7	50
	Experimental	53.1	60.5	96.9	50
	Control	57.6	59.0	57.6	50

DISCUSSION

Comparison of measurements with the literature

Hobson (1967) determined the oxygen consumption rate as a function of weight for *A. pacifica* from False Bay. According to her results, a 2-gram worm in air-equilibrated water at 12° C would consume 66 μl O_2/hr. Using equation (7) above, the value would be 84 μl O_2/hr. The comparison of the latter value with Hobson's original data of oxygen uptake versus wet weight (Hobson, 1967, fig. 5) suggests that my rates are significantly higher than those of Hobson. Seasonal acclimation to temperature by *A. pacifica* might partially explain the dif-

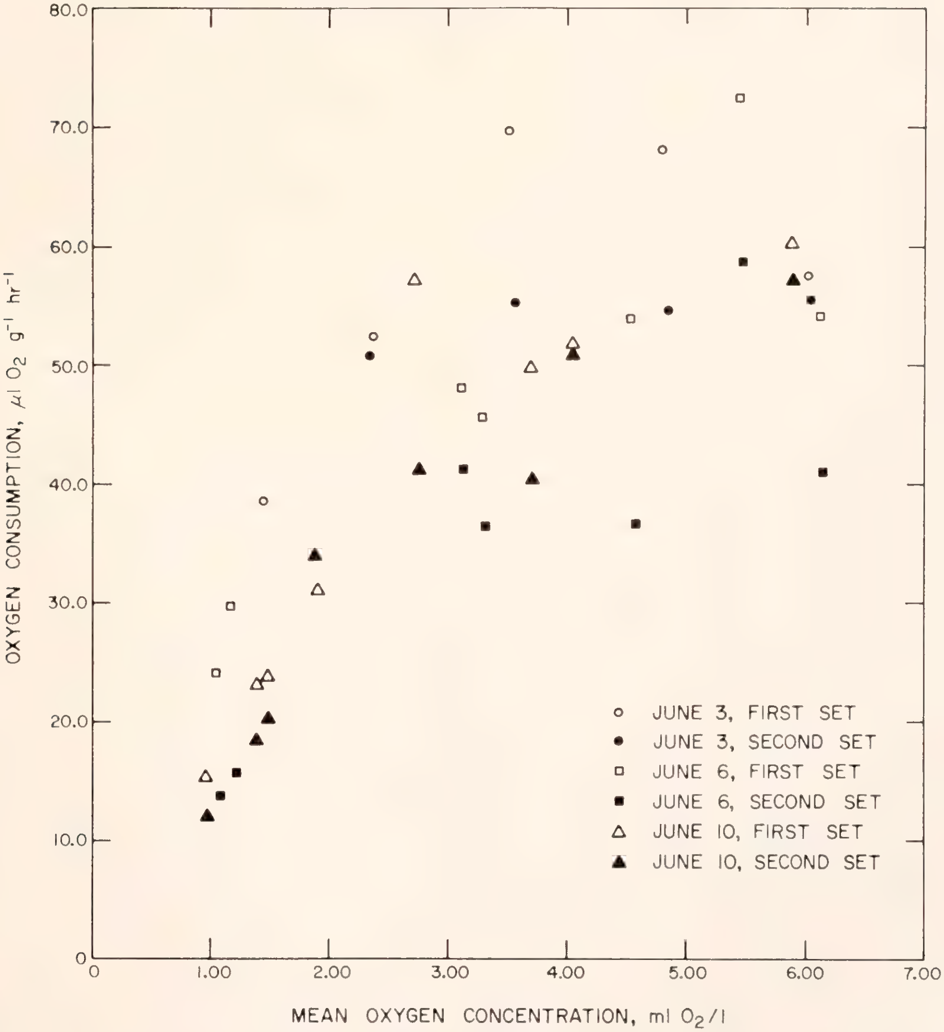

FIGURE 2. Oxygen consumption of *Lumbrineris zonata* as a function of oxygen concentration.

ference because her data were obtained in summer and fall, whereas my observations were made in late December to March.

Figure 2 shows that for *L. zonata* in the size range of 0.16 to 0.21 g, the average respiratory rate is about 50 μl $O_2g^{-1}hr^{-1}$ or 9.5 μl O_2 $worm^{-1}hr^{-1}$ for a 0.19-gram worm above 2 ml O_2/l. Using a temperature of 13° C and Pamatmat's (1968) equation for respiratory rate of *L. zonata* from False Bay in well-aerated water ($\log R = -1.51428 + 0.73710 \log T + 0.64030 \log W$), the rate for a 0.19-gram worm would be 5.8 μl O_2 $worm^{-1}hr^{-1}$, which may be compared with 9.5 above. The data of Banse *et al.* (1971) are in agreement with Pamatmat's equation above. The comparison of my observations from Figure 2 with a graph of the original data by Pamatmat and by Banse *et al.* of oxygen uptake versus wet weight suggests that my rates are significantly higher than their rates. The durations of my tests were shorter than theirs. Consequently, the higher initial rates, caused by handling the specimens, were proportionally a larger part of the tests.

Adjustment to oxygen deprivation

A. pacifica and *L. zonata* adjust to oxygen deprivation in their environment by reducing their rate of oxygen uptake. *A. pacifica* is a nonregulator at all oxygen concentrations, whereas *L. zonata* is a nonregulator below 2 ml O_2/l, above which it regulates. The reason for the difference in respiratory behavior of these two worms is not obvious. It is possible that the oxygen concentrations experienced by *L. zonata* are lower, on the average, than those experienced by *A. pacifica*. The oxygen consumption rate of *L. zonata* may be constant above 2 ml O_2/l, because it may be unable to utilize the additional oxygen available at concentrations above this level. Measurement of internal oxygen concentrations in both species, properties of their blood pigments, and their total heat production by direct calorimetry should clarify the relationship between total metabolism (aerobic and anaerobic), oxygen consumption, and external oxygen concentration. Both *A. pacifica* and *L. zonata* may regulate their metabolic rate by increasing the relative proportion of anaerobic metabolism at low-oxygen concentrations (Dr. John Machin, University of Toronto, personal communication).

Because *A. pacifica* appeared healthy after three days in anoxia, they must have metabolized anaerobically. However, I could not demonstrate the accumulation of an oxygen debt during anoxia. It is possible that they either excreted the products of anaerobic metabolism or that they repaid the oxygen debt so slowly that I was unable to detect the increase in rate after anoxia by these methods (see Dales, 1958). Because the water in their burrows was never found to be anoxic, the reason for their well-developed ability to survive anoxia is unknown. *L. zonata* also appeared healthy after two days in anoxia, and preliminary experiments indicate the presence of an oxygen debt after this time. Because the burrow walls of many worms were gray, it is possible that these worms must endure anoxia some of the time.

Some consequences of these results

Results of calculations using respiratory rates measured in aerated water will be altered significantly by the data in this paper. Hobson (1967) calculated that a 2-gram *A. pacifica* constantly in well-aerated water at 12° C would consume an average of 1.59 ml O_2/day. My calculated rate for a 2-gram worm is about 0.4 ml O_2/day. She measured the sediment turnover rate of *A. pacifica* (3.6 g sediment/day) and the average precentage of organic matter in sediment with dense populations (0.8%). Assuming a respiratory quotient of 0.8 and that one-half of the organic matter was organic carbon, she calculated the percentage removal of organic carbon from sediment (3.6%). Using a respiratory rate of 0.4 ml O_2/day, I obtain only 1.3 per cent. Both values of carbon utilization are minimum values since carbon may be used for growth, reproduction, and excretion as well as for respiration, which is a measure of maintenance only. In addition, the above calculation of carbon requirements is based only upon measurements of oxygen uptake and does not include carbon used in anaerobic metabolism, which may be important in this species. Nevertheless, the above values indicate that the food requirements of this species are low.

Banse *et al.* (1971) used data on respiratory rates of species of macrofauna and data on biomass at three stations in Puget Sound, Washington, to calculate the total respiration by macrofauna at these stations. They found that the total macrofauna at these stations only consumed between one-fifth and two-fifths of the oxygen consumed by the benthos in summer. They made all their measurements of respiration in water nearly saturated with oxygen. Because some of these species live in water low in oxygen, the actual rates of oxygen consumption are probably lower than the calculated rates. They calculated the total oxygen consumption for each species at each station. Having some knowledge of the microhabitat of each species, I could predict which species were likely to experience low oxygen levels. I then added all the oxygen consumption due to species ("major groups" only) in low-oxygen microhabitats and compared this figure to the "subtotal" in their paper, which represents all oxygen consumption by the "major groups" of macrofauna. Thus, I estimated that the minimum percentages of oxygen consumed at these stations by macrofauna likely to be living in low-oxygen microhabitats were as follows: Station 6, 40%; Station 7, 70%; and Station 11, 50%. Therefore, the relative proportion of oxygen consumed by macrofauna is probably less than that calculated in their paper.

In conclusion, I suggest that to obtain representative respiratory rates for energy flow studies involving organisms living in low oxygen, the effect of oxygen concentration on respiratory rate should be investigated.

I am grateful to Dr. Karl Banse for his support and valuable suggestions during my research. I would like to thank Dr. Robert L. Fernald for generously putting the facilities of the Friday Harbor Laboratory at my disposal. This research was supported by NSF Grant No. GB-3386 and GB-7584 to the Friday Harbor Laboratories.

Summary

1. The mean values of oxygen concentrations observed in burrows of *Abarenicola pacifica* in False Bay were 0.9 ml O_2/l at low tide and 2.3 ml O_2/l at high tide.

2. Oxygen concentrations in burrows of *Lumbrineris zonata* were not measured, but burrow walls were often gray (reduced) although sometimes tan (oxidized).

3. For *A. pacifica* with nearly empty guts, dry weight is 14.1% of wet weight, ash-free dry weight is 11.0% of wet weight, and ash-free dry weight is 77.7% of dry weight.

4. The following respiratory relationships were found for *A. pacifica:* (a) Oxygen uptake rate can be expressed as a linear function of oxygen concentration at all oxygen concentrations up to 7.0 ml O_2/l. (b) The relationship between wet weight in grams after defecation and oxygen uptake in μl O_2 $worm^{-1}hr^{-1}$ is $\log R' = 1.49 + 0.78 \log W$. (c) The relationship between oxygen concentration in ml O_2/l, wet weight, and oxygen uptake in μl $O_2g^{-1}hr^{-1}$ is $\log R = 0.93 + 0.86 \log [O_2] - 0.51 \log W$. (d) The regression of oxygen uptake on oxygen concentration, wet weight, and temperature in °C is $\log R = -0.36 + 0.86 \log [O_2] - 0.38 \log W + 1.33 \log T$.

5. During January through March, a 2-gram *A. pacifica* consumes 21.6 μl O_2/hr on the average during high tide and 3.9 μl O_2/hr during low tide or an average of 0.4 ml O_2/day.

6. *L. zonata* appears to regulate above about 2 ml O_2/l, below which it is a nonregulator.

7. *A. pacifica* is healthy after 3 days in anoxia; I was not able to determine if it incurred an oxygen debt during anoxia. *L. zonata* is healthy after 2 days in anoxia and probably incurs an oxygen debt, which it repays upon return to aerated water.

8. Calculations indicate that *A. pacifica* removes only 1.3% of the organic carbon from sediment it ingests.

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THE BENTHIC MACROFAUNA OF MORICHES BAY, NEW YORK

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The eutrophication of estuaries follows patterns that are well known in their broadest aspects but poorly known in detail. Moriches Bay on the south shore of Long Island is a textbook example (Odum, 1959) of estuarine eutrophication. While the principal cause of eutrophication here was known to be effluent from intensive duck farming along tributaries of the bay, it was aggravated by cesspool seepage and by closure between 1951 and 1954 of the only direct access to the ocean, Moriches Inlet. During this period the bay became anaerobic in summer, a situation that has been alleviated by dredging Moriches Inlet and by the gradual decline in duck farming. The bay bottom, however, contains organic sediments several feet deep in some of its more eutrophic reaches. In an effort to learn more of the details of the changes associated with eutrophication of estuaries and as a part of a longer term series of studies of the estuaries of Long Island, I have made an extensive survey of the subtidal benthic macrofauna of Moriches Bay.

Moriches Bay is a shallow lagoon located centrally along the south shore of Long Island (Fig. 1). The lagoon is separated from the open Atlantic by the Fire Island barrier beach through which one inlet is open to the sea. Channels also provide exchange with Great South Bay to the west and Shinnecock Bay to the east. The lagoon is 11.4 square miles (2963 ha) in area and averages 4 feet (1.2 m) in depth. The highly permeable drainage area of the lagoon is 80 square miles (20,720 ha).

Various aspects of the eutrophication of the bay have been examined, establishing the existence of a shift in the flora to "small forms" of phytoplankton. The blooms of these small forms was thought to have been favored by high nitrogen concentrations in reduced forms, increased temperature, and other factors (Hulbert, 1970; Ryther, 1954). High summer concentrations of these phytoplankton were correlated with the decline of commercial oyster populations in Moriches and Great South Bays (U. S. Federal Water Pollution Control Administration, 1966a, 1966b). It is also probable that reduced flushing of the bay resulted in the nuisance concentrations of the midge, *Tendipes attenuatus* (Walker) in the early 1950's (Jamnback, 1954; Jamnback and Collins, 1955, 1956).

During two successive summers oxygen deficits caused localized disappearance of macrofauna in the Forge River, the major tributary of Moriches Bay (Myren, 1964). Anaerobic conditions have been sufficiently widespread to generate enough hydrogen sulfide to discolor the paint of homes adjacent to Moriches and Great South Bays (U. S. President's Science Advisory Committee, 1965), and to cause virtually the full range of effects observed under the most severe conditions (U. S. F.W.P.C.A., 1966a, 1966b, 1967, 1968). Barlow and Myren (1961) discuss the heterotrophic nature of the Forge River in summer.

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Sediments in Moriches Bay include well-sorted sands in the shallow flats around the bay margin and extending about one-third of the distance into the bay from Fire Island (Nichols, 1964). The sands grade into clayey silts which predominate in the deeper areas. Poorly sorted sand-silt-clay and silty sand "transitional" sediments occur in zones between the bay margins and deep areas. Burrowing invertebrates cause mottling of transitional and midbay clayey silt cores. Thin shell layers occurred in many of Nichols' (1964) cores and in many benthic grabs during this study. Organic deposits derived from duck farms and from the decay of plants whose growth was stimulated by nutrients from the ducks and elsewhere. These "duck sludge" deposits are often several feet deep and contain more than 10% organic matter by dry weight (Nichols, 1964). Sandy gravel and stiff clay deposits underlie Moriches Inlet but are not found elsewhere in the bay.

Eel grass (*Zostera marina*) is the dominant vegetation, covering most of the sand flats. It has a discontinuous distribution in deeper water. The annual productivity of eel grass probably approximates the average June standing crop, *i.e.*, "maximum site" biomass, of shoots in areas where it is present (1100 g/m^2) estimated by Burkholder and Doheny (1968) in Great South Bay. A variety of attached algae are abundant only in small patches. The composition of macroalgae in Moriches is similar to that of the ajoining Great South Bay which has been surveyed by Koetzner (1963).

Benthic community structure is conventionally related to environmental parameters at several stations sampled repetitively over time. This approach permits evaluation of associations between fauna and sediment properties, current speed, *etc.* However, given data from a few purposely selected stations, it is not possible to appraise the precision with which sample observations reflect the natural communities. Thus in this study samples were selected randomly in order to embrace and estimate the spatial heterogeneity so commonly acknowledged in benthic communities (Holme, 1964).

My emphasis is, therefore, upon estimating the community structure of benthic assemblages inhabiting defined biotopes. I am much less concerned with small-scale associations between benthic forms and the milieus of particular sampling stations.

METHODS

Bottom samples were taken from April 1969 through June 1970 with a Ponar grab. This small sampler, equipped with additional weights, provided consistently deep grabs of approximately 0.05 m^2 surface area except in sandy sediments. The grab did not penetrate beyond 3 to 4 cm in sand (*c.f.* Powers and Robertson, 1967). The relatively unbiased nature of the Ponar grab has been discussed by Flannagan (1970), Hudson (1970) and Kajak (1971). Of the nine samplers in Flannagan's (1970, page 1699) detailed evaluation, he found, "The Ponar and Shipek grabs . . . came closest to being all-sediment samplers. . . ."

Sampling locations were selected by relating random numbers to coordinates of latitude and longitude, except 14 purposely selected samples mostly from dredged channels. Two replicate grabs were taken at each of 72 stations, and no station was sampled more than once. Locations of the 72 sampling stations, *i.e.*, 144 grabs, are shown in Figure 1. Samples were washed through 1-mm mesh screens. All fauna were preserved in 10% buffered formalin after relaxation in propylene phenoxetol or $MgSO_4$. Each specimen was weighed individually to three significant digits after blotting dry from preservation in formalin. Mollusc shells were not weighed. Specimens of each taxon were then stored permanently in 70% alcohol. The data on frequency and weight distribution were recorded on keypunch forms. Punched data cards were then edited by a computer program and converted to magnetic tape for subsequent analyses.

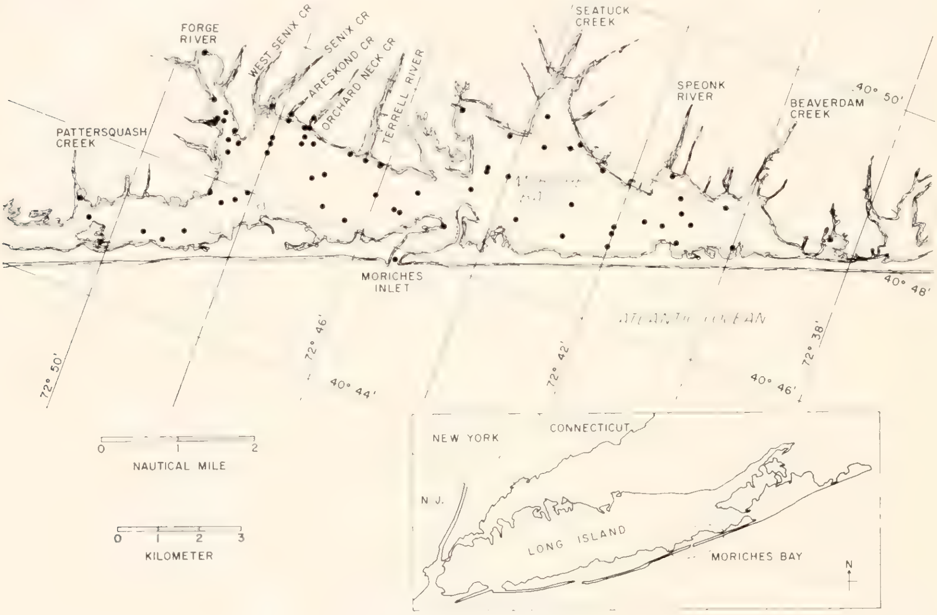
FORGE RIVER
WEST SENIX CR
SENIX CR
ARESKOND CR
ORCHARD NECK CR
TERRELL RIVER
SEATUCK CREEK
SPEONK RIVER
BEAVERDAM CREEK
PATTERSQUASH CREEK
40° 50'
MORICHES INLET
40° 48'
ATLANTIC OCEAN
72° 50'
72° 46'
40° 44'
72° 42'
72° 38'
40° 46'
0 1 2
NAUTICAL MILE
0 1 2 3
KILOMETER
NEW YORK
CONNECTICUT
N. J.
LONG ISLAND
MORICHES BAY
N

FIGURE 1. Locations of benthic samples taken in Moriches Bay, New York.

Replicated measurements of sediment redox potential were taken from one sandy and three transitional stations using a Beckman G pH meter with platinum and calomel electrodes.

Nomenclature follows Hartman (1959, 1965) for polychaetes, Abbott (1954) for molluscs, and Smith (1964) for all other taxa. Authors are provided for binomials not referenced by these authorities. Identifications were to species when possible. Of the amphipods, the Caprellidae only were identified to species.

RESULTS

The 72 samples yielded evidence of five substrates that were sufficiently different among themselves to warrant recognition as separate biotopes.

Sandy sediments make up about 37% of the bay bottom. Clayey silt and silt sediments make up about 33%. Transitional sediments, *i.e.*, sand with admixtures of from 25 to 80% clay and silt, cover about 20% of the bay bottom. Dredged channels make up the remainder, about 10%. The sandy biotope may be subdivided by distinguishing between (1) sandy sediments with dense vegetation, and (2) sand with sparse or no vegetation. Also considered separately were temporal phases of the nonsand sediments: the deeper areas with transitional, clayey slit, or silt sediments, some of which suffer prolonged oxygen depletion in summer. These potentially anoxic areas are all greater than 1 m deep, but exclude channels, and do not have appreciable tidal currents.

Bottom salinities in the open bay have ranged from 23 to 31% during the course of this study, while Moriches Inlet has remained stable since its enlargement in the spring of 1958. Salinities of less than 23% have not been encountered at any of the stations in tributaries of the bay. Bottom temperatures ranged from 0 to 27.7° C. Values above 20° C were common in July and August even in the deepest areas. Rather large areal variations in temperature and salinity result from tidal mixing and discontinuous groundwater seepage through the bay bottom. Groundwater seepage through the bay bottom is the primary freshwater source (U. S. F.W.P.C.A., 1966b).

TABLE I

Numbers of individuals per m² of benthic taxa in Moriches Bay

Taxa	Sand	Transitional	Clayey silt	Dredged channels
Amphipoda	189	1253	662	87
Decapoda	22	54	15	1
Crustacea (except Amphipoda and Decapoda)	24	25	13	17
Gastropoda	462	541	269	19
Pelecypoda	4136	486	76	6
Tunicata	—	5	50	—
Polychaeta	557	501	317	267
Turbellaria	1	<1	—	—
Nemertea	8	11	12	2
Insecta	—	—	5	—
Echinodermata	—	—	<1	—
Holothuroidea	3	5	13	—
Balanoglossida	—	1	—	—
Total	5402	2978	1433	399

Table I indicates the estimated abundance, in numbers per square meter, of the major taxa. A complete list of all benthic species encountered in 144 replicated Ponar grabs is available from: ASIS–National Auxiliary Publications Service, c/o CCM Information Corp., 909 Third Ave, New York, New York 10022. While the proportionate taxonomic representation is comparable to other studies, the absolute densities are appreciably lower than typical observations in temperate estuaries, *e.g.*, Raymont (1949), Sanders (1956), Phelps (1964), and Ellis (1967). It is also clear that dredged channels have a particularly sparse fauna in contrast with any other biotope. Murawski (1969) also found a dearth of invertebrate fauna in dredged areas of New Jersey estuaries.

The wet weights of major taxa are given in Table II. Bivalves contribute most of the wet weight biomass in sand and transitional sediments, *i.e.*, in over

TABLE II

Biomass (wet weight, g/m²) of benthic taxa in Moriches Bay

Taxa	Sand	Transitional	Clayey silt	Dredged channels
Amphipoda	1.2	3.0	1.7	0.3
Decapoda	2.1	1.6	2.0	—*
Crustacea (except Amphipoda and Decapoda)	0.3	0.3	0.1	0.1
Gastropoda	3.0	5.3	0.7	0.1
Pelecypoda	36.1	49.9	7.3	0.1
Tunicata	—	0.9	15.8	—
Polychaeta	5.5	10.6	17.7	3.5
Turbellaria	<0.1	<0.1	—	—
Nemertea	0.1	0.1	0.2	<0.1
Insecta	—	—	<0.1	—
Echinodermata	—	—	0.2	—
Holothuroidea	0.5	<0.1	0.3	—
Balanoglossida	—	<0.1	—	—
Total	58.9	71.9	46.1	4.2*

* Excludes one large *Cancer irroratus* weighing 35 g, wet weight.

57% of the bay bottom. In clayey silts the Polychaeta predominate. While tunicates contribute almost as much wet weight in clayey silt, they are much less significant in terms of dry weight or energy transfer than are polychaetes, or even bivalves.

Most ash-free dry weights were estimated by using the averages of conversion values given by Thorson (1957) and Lie (1968). Similar conversion values for tunicates were determined by the author.

The most useful illustration of biomass composition from the standpoint of carbon bound by the benthos is given by ash-free dry weights of the major taxa

TABLE III

Biomass (ash-free dry weight, g/m²) of benthic taxa in Moriches Bay

Taxa	Sand	Transitional	Clayey silt	Dredged channels
Amphipoda	0.21	0.52	0.31	0.06
Decapoda	0.36	0.28	0.35	—*
Crustacea (except Amphipoda and Decapoda)	0.05	0.05	0.01	0.02
Gastropoda	0.24	0.41	0.05	0.01
Pelecypoda	2.21	3.05	0.44	0.01
Tunicata	—	0.01	0.16	—
Polychaeta	0.89	1.71	2.86	0.56
Turbellaria	<0.01	<0.01	—	—
Nemertea	0.01	0.01	0.03	<0.01
Insecta	—	—	<0.01	—
Echinodermata	—	—	0.01	—
Holothuroidea	0.04	<0.01	0.02	—
Balanoglossida	—	<0.01	—	—
Totals	4.02	6.07	4.25	0.66*

* Excludes one large *Cancer irroratus* weighing about 6 g, ash-free dry weight.

TABLE IV

Biomass (wet weight, g/m²) of dominant species in Moriches Bay. Species listed in order of dominance by weight. Upper list contains species with average biomass ≥ 0.5 g/m² in any major sediment classification. Lower list contains less dominant species averaging ≥ 10 individuals/m² in any major sediment classification

Species	Sand	Transitional	Clayey silt	Moderate to dense vegetation	Sand w/o much vegetation	Summer oxygen depletion	Lack of, or recovery from, oxygen depletion	Dredged channels
Mercenaria mercenaria	25.5	36.3	1.6	11.3	34.0	<0.01	0.8	—
Mytilus edulis	4.7	8.2	—	8.4	<0.1	—	0.1	<0.1
Clymenella torquata	0.9	3.9	5.1	1.0	0.7	0.5	5.6	—
Bostrichobranchus pilularis	—	—	8.0	—	—	—	—	—
Molgula provisionalis	—	<0.1	7.8	0.1	—	0.1	0.1	—
Nereis succinea	0.3	0.6	4.1	0.4	0.3	1.1	1.2	—
Nassarius obsoletus	1.1	3.7	—	7.8	0.2	—	4.1	—
Glycera americana	<0.1	1.8	2.2	0.1	—	0.3	1.9	—
Gemma gemma	3.4	<0.01	<0.1	<0.1	6.0	<0.01	<0.1	—
Tellina agilis	1.3	1.4	0.3	0.7	1.8	0.3	0.6	—
Neopanope texana	1.5	0.8	0.3	3.3	0.1	—	0.7	—
Pitar morrhuana	—	2.1	2.6	—	—	—	2.7	—
Heteromastus filiformis	<0.1	0.2	1.9	<0.1	0.1	3.7	0.2	<0.1
Laevicardium mortoni	0.5	0.6	0.8	0.9	0.5	<0.1	0.4	—
Mulinea lateralis	0.1	0.4	1.4	—	0.1	0.5	1.1	0.1
Scoloplos robustus	0.4	0.6	0.7	0.5	0.3	0.9	0.3	—
Scoloplos acutus	0.6	0.2	0.3	—	0.3	0.2	0.3	<0.01
Callinectes sapidus	—	<0.01	1.5	<0.01	—	<0.1	<0.01	—
Nereis virens	0.5	—	0.5	1.0	—	1.1	—	—
Pectinaria gouldii	0.1	0.9	0.4	0.1	0.1	0.3	0.5	0.2
Urosalpinx cinerea	0.8	0.2	—	1.7	0.1	—	0.2	<0.1
Platynereis dumerilii	0.5	0.3	<0.1	1.3	<0.1	<0.1	0.3	—
Crangon septemspinosa	0.5	0.2	0.1	0.7	0.2	—	0.1	—
Botryllus schlosseri	—	0.7	—	1.1	—	—	0.8	—
Retusa canaliculata	0.1	0.4	0.4	0.1	0.1	0.4	0.4	<0.1
Capitella capitata	0.3	<0.1	0.3	<0.1	0.5	—	0.2	3.1
Nereis arenaceodonta	0.2	0.1	0.1	0.3	0.2	<0.1	0.1	—
Haminoea solitaria	<0.1	0.3	0.2	<0.1	—	0.2	0.2	—
Mitrella lunata	0.3	0.2	<0.1	0.8	<0.1	—	0.1	<0.01
Bittium alternatum	0.3	0.3	0.1	0.7	<0.1	<0.01	0.2	—
Solemya velum	0.2	0.2	0.1	0.3	—	0.2	0.1	—
Idotea baltica	0.2	<0.1	—	0.3	—	—	—	—
Hydrobia totteni	0.1	<0.01	<0.01	<0.1	0.2	—	<0.01	—
Goniadella gracilis	0.1	0.1	0.1	<0.1	0.1	0.1	0.1	—
Polydora ligni	<0.1	0.1	<0.1	0.1	0.1	0.1	<0.1	—
Polycirrus medusa	0.1	<0.1	—	<0.01	<0.01	<0.01	<0.1	—
Number of samples	23	21	17	13	13	8	19	9

(Table III). Measured as ash-free dry weight, polychaetes are relatively more important because they contain more than twice the organic carbon per unit wet weight found in molluscs. Considering the bay as a whole, polychaetes and bivalves predominate in units of ash-free dry weight, with 41 and 36%, respectively, of the total benthic dry weight.

A rather striking feature of benthic structure is the low value of average dry weight. The mean ash-free dry weight of < 5 g/m² is rather evenly distributed among the three main sediment types (Table III).

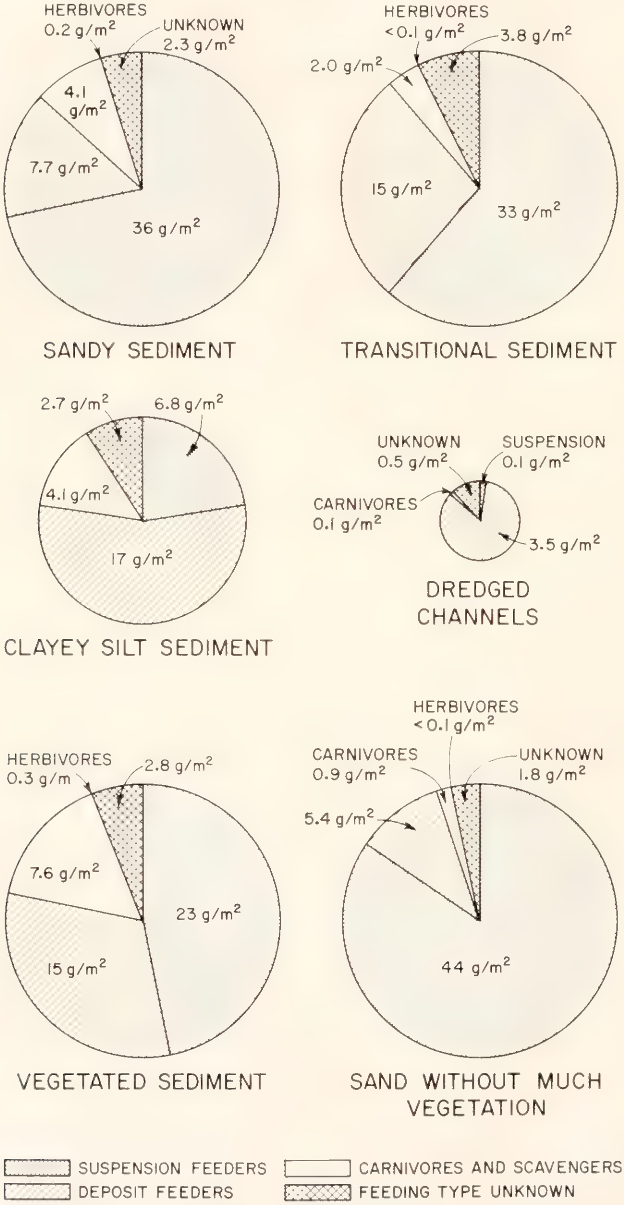

FIGURE 2. Relationships between sediment types and biomass (g/m², wet weight) of benthic feeding types. Areas of the circles are proportional to total wet weight biomass.

Table IV enumerates the biomass estimates of dominant species encountered. The large rarer specimens of the hard clam (*Mercenaria mercenaria*), blue mussel (*Mytilus edulis*) and other species induce great variability in these biomass estimates. The number of samples taken and their random selection do not insure usefully precise indications of biomass, even for the dominant species. Considerations of sampling precision will be taken up in another paper.

To help assess the influence of oxygen concentration on the benthos, a set of samples were characterized as probably suffering from prolonged summer oxygen deficits. Redox potentials in the upper 2 cm of three such stations averaged −236 mv, indicating anoxic conditions. Benthic samples taken from July through September were compared with stations of the same sediment types which either do not suffer oxygen deficits, or had some time to recover from summer oxygen depletion. While most of the latter ("recovering") stations probably underwent oxygen depletions during summer, they were sampled from January through June, thereby allowing recolonization by the benthos for periods of 4 to 10 months. All samples compared were from transitional or clayey silt sediments over 1 m deep.

TABLE V

Numbers/m² and ash-free dry weights/m² of benthic taxa from stations with, and without, low oxygen concentrations. All stations are from transitional, clayey silt, or silt sediments

Taxa	During oxygen depletion		Aerobic or partially recovered	
	No./m²	Dry wt./m²	No./m²	Dry wt./m²
Amphipoda	612	0.25	420	0.25
Gastropoda	228	0.05	527	0.43
Pelecypoda	90	0.18	79	0.36
Polychaeta	307	1.48	283	1.97
All other taxa	35	0.12	57	0.21
Total	1271	2.08	1365	3.21

Numerical abundances of the higher taxa are roughly the same at both aerobic and recovering stations, but there seems to be more biomass within the aerobic environments. Thus the individuals of most major taxa have greater average weights at the aerobic and recovering stations.

Over 90% of the wet weight biomass could be classified by feeding type. The taxa within each sediment type were defined as suspension or deposit feeders, carnivores plus scavengers, or herbivores, *i.e.*, feeding on macro-algae, eel grass or epiphytes. The weight of any species having two or more modes of feeding was equally divided among the modes. Figure 2 illustrates the marked transition from dominance of suspension feeders in the sandy sediments to dominance by deposit feeders in the fine sediments and in dredged channels.

Species diversity indices of all benthic taxa, except Amphipoda, were calculated for each biotope. The index used was derived from information theory ($H = 1/N\ (\log_2\ N! - \Sigma \log_2\ N_i!)$ where N = number of all individuals in a sample and N_i = number of the i'th species. The average H for dredged channels was 1.3 (SE = 0.41) as opposed to a mean value for the rest of the bay of 4.6 (SE = 0.25).

DISCUSSION

The low standing crop of benthos in Moriches Bay (< 5 g/m², ash-free dry weight) appears to be less than crops observed in areas with less primary production than Moriches Bay. Perhaps the most comparable area is the small coastal lagoon in Scotland studied by Raymont (1947, 1949). Estimates of primary production are apparently not available for this area, but estimates of benthic standing crop (using 1-mm mesh screens also) rose from about 4 g ash-free dry weight per square meter to a maximum of 20 g after 2 years of artificial fertilization (Raymont, 1949). The data from Sanders (1956) have been combined to estimate ash-free dry weights for all sizes of benthic forms in Long Island Sound. He appropriately warns of the variability introduced by large invertebrates, but his data are the most extensive available for the Sound. Including all species found by Sanders (1956), estimates of ash-free dry weight range from 8 to 41 g/m² (excluding extreme observations at 3 stations). In evaluating this contrast between Long Island Sound and Moriches Bay, the relative sampling bias of the Ponar grab and the modified 'Forster' anchor dredge used by Sanders must be kept in mind, but this bias has not yet been evaluated. The best estimate of annual net primary productivity in Long Island Sound is probably 43.9 gC/m² (Yentsch, 1963).

TABLE VI

Moriches Bay estimated annual net primary productivity

	Area (ha) represented	Total dry wt productivity (kg/ha/yr)	Total productivity (kg C/yr) (× 10^5)	kg C (× 10^5) in bay proper
Phytoplankton	2963	11.6×10^6	16.4*	16.4
Eel grass (shoots only)	593	6.8×10^6	24.7†	24.7
Marshes (aboveground productivity)				
Spartina alterniflora	1141	6.3×10^6	25.0†	11.3
S. patens	570	7.7×10^4	0.3†	0.1
Distichlis spicata	570	9.9×10^4	0.4†	0.2
			gC/m² in bay proper: 177.8	

* Using: kg C = 0.14 × total dry wt. (or "algal wt"). A high value within Strickland's (1960) range of conversion factors (0.09–0.15) is used because most of the Moriches Bay phytoplankton lack high concentrations of calcareous or siliceous material.

† Conversions employed the proximate analyses of Udell *et al.* (1969) and the carbon content of organic compounds given by Westlake (1963).

Any comparative estimate of annual primary productivity for Moriches Bay is imprecise, but perhaps as precise as the above estimate for the Sound. The estimates of productivity in a portion of Great South Bay by Udell, Zarudsky, Doheny, and Burkholder (1969) can be adapted to Moriches Bay for our purposes. Udell *et al.* (1969) found net annual phytoplankton productivity to be 3942 g dry matter per square meter. Where eel grass was present its net productivity was 1142 g/m², dry weight. Comparable productivity values for marsh grass stems were: *Spartina alterniflora,* 549 g/m²; *S. patens,* 502 g/m²; and *Distichlis spicata,* 645 g/m². Table VI illustrates the estimated net annual productivity of Moriches Bay proper, assuming 45% of the aboveground marsh productivity is exported to the subtidal areas of the bay as degraded organic matter and bacteria, as found by Teal (1962) in a Georgia salt marsh. The area of marsh adjoining Moriches Bay, and its dominant plant composition, was kindly provided by Mr. Roy Haje, New York State Department of Environmental Conservation, from a review of aerial photographs and correlated field surveys.

To the 178 gC/m² annual net productivity calculated in Table V must be added the carbon enrichment by duck farm effluent. Perhaps the single most useful measure of duck farm effluent is total BOD contributed to the Bay. During most of the recent life of the Bay (1940–1965) an average of about 1.4×10^6 kg BOD/yr have been flushed into Moriches Bay from duck farms (U. S. F.W.P.C.A., 1966). This is equivalent to 1.4×10^5 g BOD/m²/yr, on average, but most of this organic loading is concentrated during the summer months and it is not evenly distributed in the Bay. Since 1965 efforts have been made to reduce the organic and nutrient loading by installing aeration lagoons and settling ponds. Recently available data from Mr. Kenneth Johanson, Cornell University Duck Research Laboratory, indicates that the annual BOD loading from duck farms has been reduced to about 3.3 g/m² during 1970. Thus it seems clear that in the recent past, the benthos of Moriches Bay have had much more bound carbon available to them than the benthos of other environments discussed above.

The lower average weight of individuals at stations under oxygen stress (Table V) is consistent with the hypothesis that, throughout much of the deeper sediment summer oxygen deficits kill many benthic species or at least retard their growth rates. It is also likely that these areas with low oxygen concentrations are annually recolonized by larval forms of a few species (Table IV). It is also clear from Table IV that *Heteromastus filiformis* is the only species clearly exhibiting greater biomass in the anaerobic sediments. This species is similar anatomically and probably physiologically to the closely related *Capitella capitata. C. capitata* is widely recognized as a cosmopolitan species tolerant of, and often flourishing under, oxygen stress (Reish, 1955, 1957, 1959; Felice, 1954; Henriksson, 1969). Conversely, a number of species which are intolerant of oxygen stress are more abundant at the aerobic and recovering stations, *e.g., Nassarius obsoletus, Pitar morrhuana,* and *Clymenella torquata* (Table IV).

Despite the influence of oxygen tension, a number of additional factors almost certainly conspire to suppress benthic standing crops, perhaps synergistically with oxygen depletion. Large scale shifting of sediment was noted during the course of this study. It seems likely that such agitation and sediment deposition could destroy at least the newly settled stages of many species (Rhoads and Young, 1970). There are also substantial layers of dead shell widely distributed in deeper waters. These shell layers could interfere with the establishment of infauna, as suggested by Sanders (1956) at one of his Long Island Sound stations. Residues of DDT were found in concentrations of 0.28 lbs/acre (0.31 kg/ha) in subtidal sediments and 13.1 lbs/acre (14.7 kg/ha) in *Spartina* mats at the edge of Great South Bay (Woodwell, Wurster and Isaacson, 1967). The marshes adjoining Moriches Bay have been treated with DDT under the same mosquito control program. The impact of such DDT levels upon fiddler crabs (*Uca pugnax*) has been studied by Odum, Woodwell and Wurster (1969) and the possible selective uptake of toxins such as DDT adsorbed to clays and silts, particularly by nonselective deposit and suspension feeders, has been discussed by Carriker (1967). It is possible that DDT and other toxins have contributed to the inhibition of benthic production, particularly of carnivores and nonselective detritus feeders. The degree of predation within the benthic communities and by fishes and birds is unknown. However, Blegvad (1928) and Raymont (1947) have provided convincing evidence for measurable fish predation upon estuarine benthic communities.

The dominance of suspension feeders in sandy environments and dominance of deposit feeders in fine sediments has already been discussed by Sanders (1958), Rhoads and Young (1970), and others. Lacking information on the amount and quality of resuspended sediment, it is not possible to clearly ascribe this relationship in Moriches Bay either to a paucity of food for suspension feeders over fine sediments, as suggested by Sanders (1958) or to the several influences of instability in the biogenically reworked sediments as described by Rhoads and Young (1970).

TABLE VII

Qualitative comparison of mollusc species abundance in 1938 and 1969–70

Common to abundant in 1938, now rare	Common to abundant in 1969–70, rare in 1938
Acteon punctostriatus	*Bittium alternatum*
Eupleura caudata	
Nassarius trivitattus	
Crassostrea virginica	*Mulinia lateralis*
Mya arenaria	*Tellina agilis*
Nucula proxima	

It is clear that biogenic reworking of fine sediments in Moriches Bay is not done by the influential bivalves described by Rhoads and Young (1970) in Buzzards Bay. These species, *Nucula proxima*, *Macoma tenta*, and *Yoldia limatula*, are either rare or not found in Moriches Bay. In fact, the only two bivalves, with estimated densities greater than 15 individuals/m^2 in clayey silt are *Tellina agilis* and *Mulinea lateralis; Tellina* only being a deposit feeder. Several abundant polychaetes are undoubtedly responsible for biogenic reworking in Moriches Bay: *Clymenella torquata, Pectinaria gouldii,* and several of the Capitellidae.

Historical comparisons

A benthic survey of Moriches Bay and other Long Island estuaries is summarized by Townes (1939). Because this early survey provides only qualitative information, it is possible to detect only striking changes in benthic species composition. Differences in abundance of polychaetes are not considered because of possible confusion introduced by misclassification. It is possible to determine with reasonable certainty, however, changes in abundance of several molluscs (Table VII).

The decline of the eastern oyster (*Crassostrea virginica*) is documented from catch statistics (Nassau-Suffolk Regional Planning Board, 1966). The oyster decline is not clearly attributable to any single factor. Blooms of nannoplankton in the 1950's clogged the gills of oysters, reducing their production and market quality. However, the commercial concentrations of oysters in Moriches and Great South Bays had been maintained for many years by the commercial transplantation of spat from other areas. Commercial oyster transplantation also ceased in the late 1950's. The disease MSX was also identified as a cause of oyster mortality in the 1960's, and may have been present long before its identification. It is likely that the thick-lipped drill (*Eupleura caudata*) declined because it relied upon the oyster for food. The almost complete absence of soft-shell clams (*Mya arenaria*) is somewhat unexpected since they can tolerate rather high loads of organic matter (Henriksson, 1969). While it is possible that large individuals burrowed deeper than my grab penetrated in sand, it seems certain that many more small specimens would have been found if soft clams were abundant.

The increase in abundance of *Bittium alternatum* since 1938 is probably associated with the widening distribution of eel grass since its decline in the 1930's. Stauffer (1937) has emphaszied the close association between eel grass and *B. alternatum* on the Massachusetts coast. *Mulinia lateralis* seems more successful in clayey silt and silt sediments than is indicated in the Long Island Sound data of Sanders (1956). This fast-growing, short-lived species probably has widely fluctuating densities from year to year, particularly at specific locations.

Species diversity

Several explanations for spatial and temporal differences in benthic diversity have been summarized by Sanders (1968).

Pielou (1966) has pointed out that the Shannon and Weaver (1963) average diversity index ($H' = -\Sigma p_i \log_2 p_i$, where p_i is the proportionate abundance of the i'th species) is independent of sample size if the sampled area is truly representative of the community from which it was taken. I am not aware of any benthic studies which estimate the proportionate abundance of essentially all species in a community well enough to justify strictly the use of Shannon and Weaver's (1963) index, which may be viewed as an approximation of Brillouin's (1962) information measure:

$$H = 1/N \ (\log_2 N! - \Sigma \log_2 N_i!)$$

when each N_i (number of the i'th species) is large enough to permit use of Sterling's approximation to the logarithm of a factorial. Pielou (1966) also points out the typical problem, in practice, of bias in the average diversity (H') due to a number of small N_i, even if the proportionate abundance of rare species is accurately estimated.

Thus H was calculated (as preferable to H') for several biotopes of Moriches Bay. Exact values of $\log_2 N!$ and $\log_2 N_i!$ were calculated for values of $N \leq 175$; Sterling's approximation being used for larger values. Comparisons were made between means of sample H values from sand, transitional, clayey silt, summer samples from clayey silt and transitional areas with summer oxygen depletion, samples from these areas after at least four months of recovery from oxygen deficits, and dredged channels. The Student–Newman-Keuls test for multiple comparisons (Sokal and Rohlf, 1969, pages 242–246) was used to test for differences between means of H from these biotopes. The diversity of dredged channels was significantly less ($P < 0.05$) than each other biotope, but no significant differences were detected between any two of the other biotopes.

This inability to detect significant differences in species diversity among sediment types raises an important question. How powerful is the significance test used? That is, what is the probability of finding biologically meaningful differences in diversity if they really exist? This risk, or error, was evaluated using:

$$n \geq 2\,(\sigma/\delta)^2\ \{t_{\alpha[v]} + t_{2(1-P)[v]}\}^2$$

as given by Sokal and Rohlf (1969, pages 246–249) where: n = number of replicates; σ = true standard deviation $\simeq$ s = 1.81 in this study; δ = the smallest true difference that it is desired to detect, taken to be 1.38 in this study, *i.e.*, the observed difference between means of H in transitional and clayey silt biotopes; v = degrees of freedom of s = 59 in this study; $P\ (=\beta)$ *i.e.*, the desired probability that a difference as small as δ will be found to be significant; $t_{\alpha[v]}$ and $t_{2(1-P)[v]}$ = values from a two-tailed t table with v degrees of freedom and corresponding to probabilities of α and $2(1-P)$, respectively. Given the effort in this study of 62 replicated samples, the β error is $> 60\%$, *i.e.*, the probability is less than 0.4 that we will detect (at $\alpha = 0.05$) a difference between two sediment types of $\Delta H = 1.38$ which is presumed to exist. To reduce this β risk to 0.2, it would have been necessary to take 84 replicated samples.

As in most ecological field studies, my limitation to 62 replicated samples was dictated almost entirely by the practical consideration of available manpower, not by *a priori* assessment of the likelihood of answering particular questions. This is a serious flaw in many ecological studies. It is clearly evident in this study, if a real difference of 1.4 between the diversity of two biotopes is judged biologically meaningful. Thus it seems likely that if benthic studies are to critically assess species diversity (and other structural parameters of communities) they must employ appreciably more sampling effort than is typical of benthic studies. (The further importance of well-designed sampling strategies is even more evident.)

Because of obvious differences among studies such as various screen mesh sizes, sampling gear, *etc.*, it is not possible to compare the species diversity of Moriches Bay with most other studies. Differences in sampling strategy also make comparisons difficult or impossible; the expected variance of H from randomly selected samples, as taken in this study, will be higher than the variance of repeated samples from a few fixed stations. Indeed, it is typically impossible to compare with any confidence the diversity of samples from natural communities, given repeated samples at purposely-selected stations, because the sample variance will inevitably underestimate the population variance.

Despite the lack of comparability between this study and all others it seems likely that the species diversity of Moriches Bay is somewhat higher than that of a Rhode Island coastal lagoon studied by Phelps (1964). Phelps' diversity values (calculated as H′) averaged 2.1 in "mud" and 2.8 in sand. Since his sample sizes (0.13 m^2) were essentially the same as used in this study, and since he used 0.5-mm mesh screens as opposed to the 1-mm mesh used here, his expected sample values of H would average larger than mine if the two areas had the same species composition. Phelps also identified species of all amphipods, whereas I identified only the caprellids, which should even further increase his diversity estimates. Thus, Moriches Bay diversity values ($\overline{H} = 4.6$) are significantly higher ($P < 0.001$, using Student's t test) than those of Phelps (2.4) if the sample variance of H in Phelps' lagoon is identical to that of Moriches Bay.

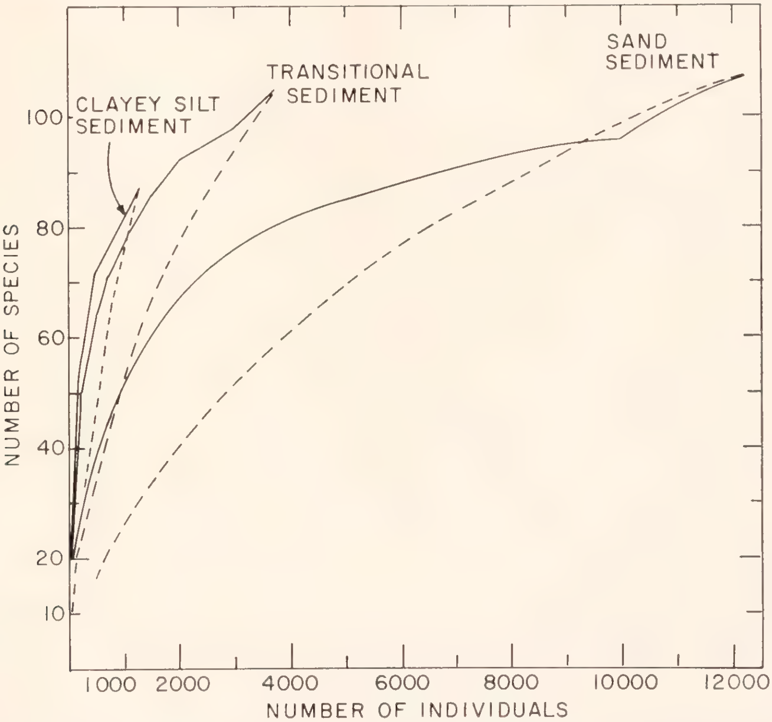

FIGURE 3. Rarefaction curves (———) and empirically derived "species/individuals" curves (– – – –) for all taxa sampled in Moriches Bay. Amphipods, except Caprellidae, are not included.

Sanders' (1968) rarefaction index of species diversity has also been calculated for all species encountered in Moriches Bay (Fig. 3). It must be noted that the algorithm given by Sanders (1968) for his rarefaction curve results in higher numbers of species at almost all frequencies of individuals than are actually experienced in sampling. This is because Sanders' rules for generating rarefaction curves result in consistent overestimates of the numbers of species encountered at all levels of sampling intensity except near both extremes of a rarefaction curve. This feature of rarefaction curves does not rule out their use as indices of diversity, but the curves do not strictly portray the regression of numbers of species encountered on numbers of individuals sampled. Thus, as shown in Figure 3, the rarefaction curves are consistently above the corresponding curves calculated from the averages of species and individuals found in all possible combinations of samples taken 1, 2, 3, *etc.*, at a time. These latter curves, are identical with the classical "species area" curves widely used in ecology; the units of the abscissa in Figure 3 are simply average number of individuals per (cumulative) unit of area. The units of the abscissa could as well be cumulative area sampled, without changing the coordinates of the curve at all. Thus the curves of Figures 3 and 4 are called "species/individuals" curves without any implication that they are yet another diversity measure. It is also clear that neither these curves nor rarefaction curves are independent of sample size

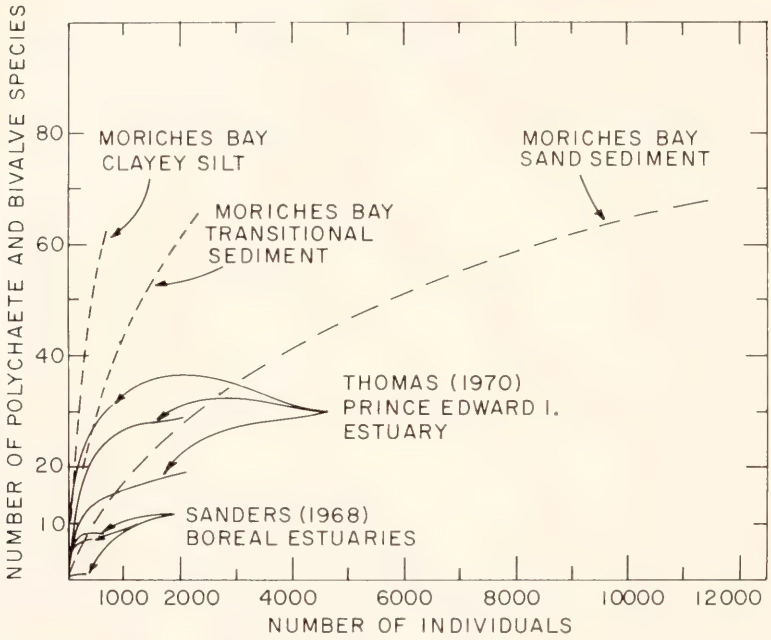

FIGURE 4. Rarefaction curves (———) and empirically-derived "species/individuals" curves (– – – –) for bivalves and polychaetes.

(Greig-Smith, 1964, page 151). While techniques are not available for statistical comparison of either measure of diversity shown in Figure 3, most of the difference between the curve for sand sediments and those of other sediment types is due to the preponderance of *Mytilus edulis* and *Gemma gemma* in sand samples (9686 individuals, or 79% of all specimens from sand samples).

Since Sanders' (1968) rarefaction curves, and those of Thomas (1970) include bivalves and polychaetes only, a comparison of rarefaction and "species/individuals" curves for these taxa is given in Figure 4. The Moriches Bay curves in this figure are much steeper than those of both Sanders and Thomas from boreal estuaries, primarily because my study employed random sampling of species almost always contagiously distributed, in contrast to the repetitive sampling at fixed stations by Sanders (1968) and Thomas (1970). It is important to note that the rarefaction method was designed as a measure of "within-habitat" diversity (Sanders, 1968, page 243). That is rarefaction curves have been generated by Sanders and others from samples within rather homogeneous species assemblages. Rarefaction curves will differ from "species individual" (or species/area) curves less as the sample universe becomes more homogeneous. Indeed, when all samples are from essentially the same location, the resultant species/individuals and rarefaction curves should differ very little. Sanders and his colleagues are currently exploring the question in more detail. Thus no diversity comparisons are made between this study and the diversity of Buzzard's Bay studied by Sanders, or of Bideford River, Prince Edward Island, studied by Thomas.

Unsatisfactory results were found in attempting to trace the seasonal growth patterns of even dominant species. While this technique has been used by Sanders (1956) to estimate benthic productivity and by Lie (1968) to estimate the growth rates of some benthic species in Puget Sound, I was unable to estimate growth rates of any species. Given the sample-processing effort expended in this study (about one man-year) I question the desirability of expending the effort required to estimate species-specific productivity rates of the benthic macrofauna by this method in species-rich estuaries.

All the data gathered in this study are available on magnetic tape from the National Oceanographic Data Center, Rockville, Maryland. Included in these data files are codes indicating the feeding types assigned to species.

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SUMMARY

Quantitative sampling of the subtidal macro-benthos of a shallow temperate lagoon shows several structural features of benthic communities in five biotopes. Attempts are made to relate past and present qualities of benthic structure to long-term nutrient and organic loading of this lagoon. While overall benthic species composition has apparently not changed drastically since a 1938 qualitative survey, some important changes in dominant species composition appear to be associated with the biology of these principal species. The best estimate of average annual standing crop of the macro-benthos (< 5 g/m^2, ash-free dry weight) is lower than estimates in somewhat comparable estuaries, apparently because of low oxygen concentrations over extensive areas during summers, which derive in turn from large summer influxes of nutrients and organic matter into this poorly flushed lagoon. Clear-cut associations between sediment type and mode of feeding of the benthos are demonstrated; suspension feeding invertebrates dominating in sandy sediments, and deposit feeders dominating in soft clayey silt bottoms. Species diversity appears to be homogeneous over most of the bay, with very low diversity in dredged channels. The need for more, and

more carefully allocated, sampling effort in benthic studies is emphasized, given the imprecision in community structural features, particularly species diversity and biomass of genera and species.

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DETERMINATION OF FOOD PREFERENCE OF *STENTOR COERULEUS*

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Schaeffer (1910) found that the ciliate protozoön, *Stentor coeruleus,* selected among the particles that were brought to its buccal cavity (by its adoral membranelles). Some particles were preferentially rejected by a localized ciliary reversal while others were carried to the cytostome and ingested. Selection was hypothesized not only among the particles reaching its buccal cavity successively, but also among particles reaching the cavity at the same time. Furthermore, the amount ingested depended upon the other substances present. For example, Schaeffer found that carmine particles, although indigestible, were ingested by stentors in the absence of food organisms, but rarely taken when food organisms were present. Stentors were found to discriminate more perfectly when almost satiated than when very hungry, since hungry individuals ingested particles such as carmine and india ink. Schaeffer found that this species also discriminated between different types of organisms, ingesting some (*Euglena* sp., *Phacus triqueter*) with great readiness, while others (*Trachelomonas hispida, Phacus longicaudus*) were rarely ingested.

Hetherington (1932) reported that on the basis of an extensive series of trials, *S. coeruleus* ingested various autotrophs "sparingly" when hungry. The autotrophs tested included *Gonium pectorale,* several species of *Euglena, Trachelomonas,* and diatoms. In contrast, stentors were found to ingest "avidly" many of the ciliates tested. Hetherington suggested that *S. coeruleus* had a "general preference" for ciliates and was capable of selection even within this group. Tartar (1961) reviewed these studies as well as others and concluded that "on the evidence, food selection does occur in *Stentor,* though by no means perfect and distinctly related in its acuity to the state of the organism."

Such studies, however suggestive, fail to account for differences in "prey catchability" or provide a measure of the statistical significance of preferences. The work reported here makes use of a new definition of food preference (Rapport and Turner, 1970) which lends itself to a determination of preference in the predator-prey context without confounding differences in prey catchability. The method involves comparing the mean number of prey consumed when each prey species is present alone with the mean number of prey of each species consumed when several species are present at the same time.

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MATERIALS AND METHODS

Stentor coeruleus was collected from Grenadier Pond, Toronto, Ontario and a clone was established for the purpose of these experiments. It was maintained according to culture methods used by Tartar (1961). Eighteen to 24 hours prior to the experiment, stentors were removed from culture and placed in new cultures with reduced levels of prey organisms which served to induce a level of starvation which insured an adequate feeding response to prey offered during the experiment.

TABLE I

Mean number of prey consumed per stentor in single and mixed culture feedings. Tetrahymena pyriformis (*T*), Chilomonas paramecium (*C.p.*), Euglena gracilis (*E*), *and* Chlamydomonas reinhardti (*C.r.*)

Prey species	Replicate											Mean
	1	2	3	4	5	6	7	8	9	10	11	
C.p.	16.44	14.24	19.48	25.09	31.26	N.A.*	22.88	14.64	26.44	14.04	12.39	19.69
C.r.	47.32	45.60	58.64	67.88	86.92	283.04	182.64	149.92	153.04	76.78	52.52	109.50
E	22.04	12.28	10.76	27.84	18.52	14.42	9.60	11.44	6.28	5.16	11.74	13.64
T	9.76	4.92	11.56	12.40	13.00	11.18	10.96	17.48	14.84	5.86	6.60	10.77
C.r.	27.96	30.76	49.48	57.68	91.12	114.50	61.88	64.44	34.08	23.32	97.86	59.37
with												
C.p.	10.48	20.00	20.48	21.08	31.92	28.18	12.00	40.92	13.52	10.48	30.23	21.75
C.r.	49.16	49.68	39.60	34.12	139.56	87.76	158.16	94.72	40.50	29.96	22.16	67.76
with												
E	9.32	15.44	5.60	8.12	10.40	20.16	18.28	3.44	3.13	6.70	17.08	10.69
C.r.	7.68	23.08	12.00	12.80	20.12	110.76	44.08	14.76	29.76	6.43	10.20	26.51
with												
T	8.36	12.52	9.00	4.12	12.52	16.36	18.67	5.60	17.40	17.64	11.40	12.14
C.p.	13.68	13.04	16.52	23.16	31.44	11.88	33.64	22.08	5.92	22.43	8.26	18.36
with												
E	8.04	2.52	4.20	4.72	8.40	16.92	10.68	2.84	2.36	5.43	3.00	6.28
C.p.	12.08	12.16	13.20	13.76	11.84	22.92	12.08	41.09	13.72	5.00	8.48	15.12
with												
T	8.76	6.04	5.00	6.16	4.80	12.44	7.20	17.73	7.88	1.33	1.38	7.15
E	4.56	15.64	2.16	9.76	6.04	10.65	0.36	5.20	7.00	4.89	20.72	7.90
with												
T	6.32	10.76	7.24	15.96	10.24	9.35	3.80	16.60	7.26	9.11	8.92	9.59

* Not available due to inadequate fixation.

After stentors were washed gently 3 times in millipore-filtered pond water (0.2 μ pore) to remove bacteria and other organisms which serve as food in cultures, they were then transferred in one ml of millipore-filtered pond water to an embryological block cell. Solid embryological block cells of 4 ml capacity with a true hemispherical cavity were obtained from P. K. Dutt and Co. Ltd., Bromley, Kent, England. Prey species were added and stentors were allowed to feed for a period of twenty minutes. The choice of feeding period (20 minutes) enabled most stentors to capture prey but not digest them to the stage where prey recognition becomes difficult. Subsequently, stentors were washed 3 times in sterile pond water to remove adhering prey and then were fixed in a weak solution of formalin. Several drops of 6% formalin were added to 10 ml of millipore-filtered pond water containing washed stentors. Stentors in this state remained well preserved for several weeks. During this period, each stentor was examined individually using light microscopy and the number of each prey species ingested was noted. As food vacuoles containing prey often were obscured by each other it was necessary to compress a stentor gently under a coverglass in order to count all prey present.

Four prey species, *Tetrahymena pyriformis,* strain GL, *Euglena gracilis, Chilomonas paramecium* and *Chlamydomonas reinhardti,* representing protistan genera commonly in fresh water ponds in which *S. coeruleus* can be found, were used in these experiments. Prey were washed several times by alternative gentle centrifugation and resuspension in millipore-filtered pond water and subsequently examined microscopically for damage due to washing. The following prey densities were found to be appropriate for the preference tests: *Tetrahymena pyriformis* 10,000/ml, *Euglena gracilis* 15,000/ml, *Chilomonas paramecium* 30,000/ml, *Chlamydomonas reinhardti* 60,000/ml. These densities approximated the "standard densities" required to determine predator food preferences (Rapport and Turner, 1970). Standard density is defined as the minimum density of prey such that the predator would be able to fulfill its food requirements from any single species alone in the mixed prey environment.

The axenic cultures of prey organisms were obtained from the following sources: *Tetrahymena pyriformis* from Joel Hermolin, University of Toronto, *Euglena gracilis* from L. Cohen, York University, *Chlamydomonas reinhardti* from E. Rapport, York University, and *Chilomonas paramecium* from H. S. Ducoff, University of Illinois.

The experimental design consisted of 11 treatments and 11 replicates with a sample size of 25 stentors in each treatment consisting of either zero (control), one, or two prey species, each in their standard density. The entire experiment was carried out in a controlled temperature room at 18 ± 1° C.

In order to calculate preference, one must have an estimate of μ_1, the mean number of prey species 1 consumed in a standard time interval in single culture; μ_2, the mean number of prey species 2 consumed in single culture; μ, the mean number of prey consumed in mixed culture; μ_1^*, the mean number of prey species 1 consumed in mixed culture and μ_2^*, the mean number of prey species 2 consumed in mixed culture. In the absence of preference, the predator achieves its food requirements half from prey species 1 and half from prey species 2. Thus:

$$\mu = \frac{\mu_1}{2} + \frac{\mu_2}{2}.$$

If preferences are exercised, the mean number of mixd prey taken can be written:

$$\mu = \frac{p_1\mu_1}{2} + \frac{p_2\mu_2}{2}$$

The parameters p_1 and p_2 are preference coefficients and can be computed as follows:

$$p_1 = \frac{2\mu_1^*}{\mu_1}; \quad p_2 = \frac{2\mu_2^*}{\mu_2}$$

The relative preference $p_{1,2}$ denotes preference for prey species 1 if its value is positive and prey species 2 if negative. It is defined by: $p_{1,2} = p_1 - p_2$.

Observations and Results

The mean prey consumption data are given in Table I. It can be seen that the mean consumption differed among the prey species, ranging from 10.7 in the case of *Tetrahymena pyriformis* to 109.5 for *Chlamydomonas reinhardti*. This difference reflects both the difference in average size of the prey species (as shown in Table V) and the difference in the "standard" density appropriate for each prey species. The variance between replicates demonstrates the need for a large number of replicates in order to obtain statistically significant results. In Table II, the relative preference values are given as calculated from the basic data in Table I according to the methods described above. In the absence of preference and allowing for sources of variance, we would expect that on average, an equal number of preference coefficients would be positive and negative. Significant deviations from the no-preference case were observed in all cases in which algal and non-algal prey were paired.

Table II

Relative preference ($p_{1,2}$) *of* Stentor coeruleus *for selected pairs of prey species*

Prey species		Relative preference Replicate #										
(1)	(2)	1	2	3	4	5	6	7	8	9	10	11
T	C.r.	1.4	4.1	1.1	0.3	1.5	2.1	2.9	0.5	2.0	5.9	3.1
T	E	0.9	1.8	0.8	1.9	0.9	0.2	0.6	1.0	−1.2	1.2	−0.8
C.p.	C.r.	0.1	1.5	0.4	0.0	0.0	N.A.*	0.4	4.7	0.6	0.9	1.1
C.p.	E	0.9	1.4	0.9	1.5	1.1	N.A.*	0.7	2.5	−0.3	1.1	0.8
C.p.	T	−0.3	−0.7	0.5	0.1	0.0	N.A.*	−0.3	3.6	0.0	0.3	1.0
C.r.	E	1.2	−0.3	0.3	0.4	2.1	−2.2	−2.1	0.7	−0.5	−1.8	−2.1

* Not available due to inadequate fixation.

The results of the statistical analysis of these data are shown in Table III. It is apparent that *S. coeruleus* prefers *Chilomonas paramecium* when paired with either *Chlamydomonas reinhardti* or *Euglena gracilis*, and prefers *Tetrahymena pyriformis* to *Chlamydomonas* and *Euglena*. (Although P exceeds 0.05 in the case where *Tetrahymena* was paired with *Euglena*.)

In contrast there was no evidence of significant preferences between *Chilomonas* and *Tetrahymena*, or *Euglena* and *Chlamydomonas*.

Table IV presents the data on average consumption in mixed prey feedings as a percentage of the average consumption in single prey feedings. In each case,

TABLE III

Food choice of Stentor coeruleus *when two prey species are simultaneously available.* Tetrahymena pyriformis (*T*), Chilomonas paramecium (*C.p.*), Euglena gracilis (*E*) *and* Chlamydomonas reinhardti (*C.r.*) *were used as prey. Each replicate consisted of three groups of 25 stentors. One group was fed both prey simultaneously, while the other two groups were fed single prey species*

Prey species		Number of replicates	Number of replicates in which prey 1 preferred	Probability*
(1)	(2)			
T	C.r.	11	11	0.001
T	E	11	9	0.07
C.p.	C.r.	10**	9	0.02
C.p.	E	10**	9	0.02
C.p.	T	10**	6	N.S.***
C.r.	E	11	5	N.S.***

* Probability of obtaining results by chance alone if no real preference exists. These probabilities were calculated as the exact probability of obtaining no more than the percentage of minority results assuming that the distribution of relative preference is binomial and that preference could be for either prey. The calculation is made by summing the appropriate number of terms of the binomial expansion. Similar results were obtained by use of T-test statistics.

** Data from one replicate was not obtained due to inadequate fixation.

*** N.S. Not Significant. Probability of obtaining results by chance >0.10.

stentors reduced their consumption of the non-preferred species by a greater amount than the preferred species. These data show that the population of stentors did discriminate between algal and non-algal prey species. When non-algal prey were paired with algal prey in mixed culture, stentors took approximately the same quantity of the non-algal prey consumed in single culture, while taking only one-quarter to one-half the amount of the algal prey consumed in single culture. When the two algal species were paired, or when the two non-algal species were paired, stentors consumed about three-quarters of the quantities of each prey species consumed in single culture (within the limits of experimental error). Thus although the stentors increased its total prey consumption by approximately 50% in the mixed-prey feedings, the increase favored the consumption of non-algal

TABLE IV

Average prey consumption in mixed prey feeding as percentage of single prey feeding consumption

	Euglena	*Tetrahymena*	*Chlamydomonas*	*Chilomonas*
*Euglena**	—	57%	78%	46%
Tetrahymena	89%	—	112%	66%
Chlamydomonas	61%	24%	—	54%
Chilomonas	93%	76%	110%	—

* Reference species are shown in the 1st column. For example, in the mixed feeding of *Euglena* and *Tetrahymena*, stentors consumed 57% of the number of euglenae taken in single culture.

prey when paired with algal prey, while being drawn more equally from both populations when algal prey or non-algal prey were paired.

The increase in the amount consumed when both prey are present may be attributed to an increase in total prey density suggesting that the prey densities chosen for the experiment were somewhat below the "standard density." Thus stentors may have consumed some non-preferred prey in part because they were not fully satiated with the quantities of preferred prey that they could capture. It is also possible that there is a high cost of sorting out the non-preferred prey in cases where both prey are simultaneously brought to the "selection site."

DISCUSSION

Our findings are consistent with Hetherington's speculation that *Stentor coeruleus* has definite preferences for non-algal prey over algal prey. Considering all four prey species, *S. coeruleus* demonstrates a remarkably consistent preference pattern.

TABLE V

Size ranges of species used as prey

Prey	Length (μ)
Tetrahymena pyriformis (Corliss, 1953)	34–74
Euglena gracilis (Gojdics, 1953)	31–53
Chilomonas paramecium (Kudo, 1966)	30–40
Chlamydomonas reinhardti (Levine, 1960)	8–15

As Schaeffer (1910) has indicated, the degree of preference may vary in part with the hunger state of *S. coeruleus*. This factor may account for some of the variance in the relative preference values obtained. We attempted to control the hunger state of the predator by placing all stentors in a standardized feeding condition for 24 hours prior to the beginning of the experiment. However, since the first and last replicates were done approximately 14 hours apart, hunger states may have indeed varied between replicates. Other factors such as cell cycle may have also affected their feeding response.

It is of interest to note that preferences were not correlated with differences in prey densities used in the experiment, nor were they correlated with differences in prey size as shown in Table V.

The existence of food preferences at the protozoan level has been documented for *Stentor coeruleus*. Using other definitions, food preferences have been reported for many "higher" organisms, both invertebrates and vertebrates (Murdoch 1969, Thompson, 1965). To the extent that food preferences are found in all animal phyla, food preferences would appear to be of fundamental adaptive significance for organisms. To the extent such preferences correlate with the "welfare" of the predator, preference may explain "predator switching" and changes in predator strategies from energy maximizers to time minimizers (Rapport, 1971).

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SUMMARY

Four protistan species, *Tetrahymena pyriformis, Chilomonas paramecium, Euglena gracilis,* and *Chlamydomonas reinhardti,* were fed individually or in pairs to the ciliate *Stentor coeruleus.* Making use of a new definition of food preference which does not confound catchability with choice, this species' food preferences were measured by comparing the mean consumption of a population of stentors when each prey species was present alone with the mean consumption when a pair of prey species was present. *S. coeruleus* was found to exhibit consistent food preferences, preferring protozoan to algal prey while indicating no preference when choosing between algal or between protozoan prey. Preferences were not correlated with prey size.

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BEHAVIORAL AND ELECTROPHYSIOLOGICAL STUDIES OF HYDRA. III. COMPONENTS OF FEEDING BEHAVIOR[1]

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The mechanisms controlling feeding responses in coelenterates were the subject of much research at the turn of the century (Nagel, 1892; Loeb, 1895; Jennings, 1905; Parker, 1896, 1917). Recently interest in coelenterate feeding behavior was rekindled by a report that in the freshwater *Hydra*, a feeding response is specifically activated by reduced glutathione (GSH) (Loomis, 1955). This finding led to extensive study of the effects of this tripeptide as a feeding stimulator in *Hydra* (Lenhoff, 1961a, 1961b, 1965, 1967, 1968a, 1968b), and a search for other simple molecules activating feeding in other coelenterates (Fulton, 1963; Mariscal and Lenhoff, 1968; Linstedt, Muscatine and Lenhoff, 1968; Pardy and Lenhoff, 1968). The uniqueness of GSH as an initiator of feeding in *Hydra* was questioned both by Forrest (1962) and Burnett, Davidson and Wirnick (1963). However, many of their questions have been answered in Lenhoff's later experiments (Lenhoff, 1965, 1968a).

In the past decade, there has been a mounting interest in electrophysiological studies of hydroids (Josephson, 1961, 1965a, 1965b, 1967, Josephson and Mackie, 1965; Passano and McCullough, 1962, 1963, 1964, 1965; Rushforth, 1967, 1971; Mackie, 1968). These investigations have characterized, in considerable detail, the properties of pacemaker activities underlying stereotyped, rhythmically recurring behavioral events. Electrical recording techniques have given us considerable insight into the types of potentials associated with spontaneous behavior in several hydroids. There is little information, however, concerning the electrical correlates of feeding responses in these animals. The present research was undertaken to study sequences in the feeding behavior in *Hydra* and to determine if changes in electrical activity accompanied such sequences. Similar studies were performed on the marine hydroid *Tubularia* for comparative purposes (Rushforth, in preparation).

Materials and Methods

Most of the experiments were performed using *H. littoralis*, the animal most extensively used by Loomis and Lenhoff in their investigations of feeding behavior. However, since previous papers in the present series have investigated the electrical activities of *H. pirardi* and *H. pseudoligactis*, these two species were also

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used, particularly in the electrophysiological studies of feeding responses. All animals were cultured using methods previously described (Rushforth, 1971).

The animals used had been fed 24-hours previously with *Artemia salina* nauplii. Since Lenhoff (1965) showed that the feeding response in *Hydra* was influenced by the microenvironment surrounding the animal, particularly in crowded cultures, an effort was made to thoroughly wash the hydra before making observations. Individual hydra were rinsed by pipetting successively in three finger bowls each containing 200 ml of buffered culture solution. The animals were then placed in a fourth finger bowl, also containing 200 ml of the same solution (1.5×10^{-3} M $CaCl_2$, 1.2×10^{-3} M $NaHCO_3$ and 1.2×10^{-4} M Na_4 EDTA and 10^{-3} M histidine buffer, pH 6.2), and were left undisturbed for an hour before the start of an experiment.

Single nauplii were sucked into a fine glass holder made from a pasteur pipette. One end of the pipette was drawn out into a fine bore, the other end being attached to flexible plastic tubing. By releasing the suction gradually, the nauplius was gently directed onto a selected region of a given tentacle. By means of stop-watches the times for various resulting behavioral sequences were recorded. All observations were made using a dissecting microscope. An extract of *Artemia salina* was prepared from a dense 1-ml suspension of nauplii in 5 ml of distilled water. The preparation was homogenized in a small tube using a ground glass plunger, and the resulting suspension was centrifuged at 3000 rpm for 15 minutes. Aliquots of 1 ml of the supernatant, approximate total protein content of 6 mg/ml, were diluted ten fold with culture solution and a sample of 1 ml of this solution was administered to the hydra.

Electrical recordings were made from individual hydra using methods previously described (Rushforth, 1971). The animal's movement was only restricted by its base being attached to the recording electrode. Single *Artemia*, homogenates of *Artemia*, or GSH were pipetted into the environment of the animal while its behavior and electrical activity were recorded. Minor modifications of these experimental procedures will be described in the appropriate section in the presentation of the results.

Results

The most concise description of the feeding response of *Hydra* was given by Josephson: "First the prey strikes the polyp, usually on one of the outstretched tentacles, and becomes there attached by nematocyst discharge. The portion of the tentacle proximal to the prey then contracts, often spiralling inward, which brings the prey near the mouth. As the prey nears the mouth, the surrounding tentacles concertedly flex in the oral direction. This sometimes results in adjacent tentacles contacting the prey and pushing it towards the mouth. Concerted tentacle flexion may be repeated several times during and after ingestion of the prey. Finally the mouth opens, creeps around the prey, and closes about it" (Josephson, 1965c, page 34).

In the present study we have investigated: (1) tentacular movements following nematocyst discharge; and (2) inhibition of the normal endogenous contraction activities of the tentacles and body column, which takes place concurrently with the reflexly-linked, local responses of feeding.

1. *Tentacular movements*

On attachment of the *Artemia* to the tentacle by nematocyst discharge, there is a latent period before the first observable movement of the tentacle. This time period, measured in seconds, we define as the tentacle reaction time. The tentacle movement usually consists of contraction proximal to the point of contact of the *Artemia,* but there may be bending or spiralling movements associated with the shortening, depending on the position of the attachment. Such activities of the stimulated tentacle bring the prey near to the mouth and precede a concerted flexion of the group of tentacles (termed a concert). As such concrts repeatedly occur, the mouth opens and begins to creep around the prey. At this stage the concerted tentacular movements often are replaced by uncoordinated writhing activities. Such writhing movements of the tentacles have been reportd by several other workers (Ewer, 1947, Loomis, 1955, Lenhoff, 1961b). The mouth continues to creep around the *Artemia,* finally closing about it. The time in

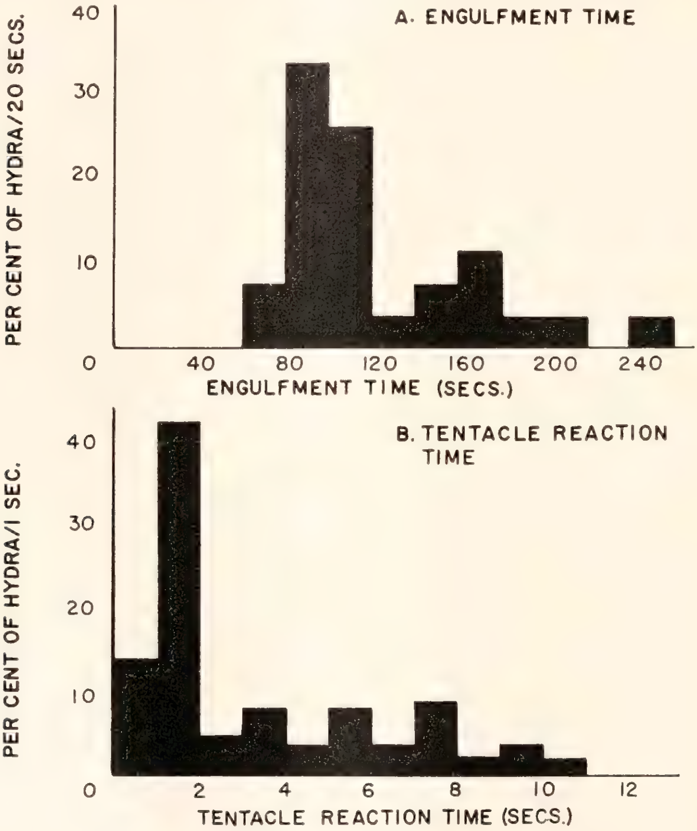

FIGURE 1. Distributions of engulfment and tentacle reaction times for *H. littoralis;* A: histogram of the per cent distribution of engulfment times for a group of 27 hydra; B: histogram of the per cent distribution for tentacle reaction times for a group of 102 hydra.

seconds from prey attachment to mouth closing we have termed the engulfment time. The distributions of engulfment times (A) and the reaction times (B) for *H. littoralis* are given in Figure 1. Both distributions are skewed to the right with modal class intervals of 80–100 seconds or 1–2 seconds, respectively.

The tentacle reaction initiated by *Artemia* capture depends on the part of the tentacle involved in the capture. This was systematically examined by arbitrarily subdividing the tentacle into three portions: the basal; middle and distal regions. Each of the three regions was tested with each of 30 hydra, the order of observing the regions was selected at random. The results are shown in Table I. They indicate the widę range of tentacle movement used to bring the prey organism to the mouth of the hydra. The fact that contraction of the tentacle is limited to the portion below the point of prey attachment, strongly suggests polarized conduction in the oral direction. The three forms of tentacle movement may result primarily from mechanical stimulation by the prey, but they do not occur without nematocyst discharge. If a clean glass rod is lightly touched to the tentacle, there is only slight localized contraction at the point of stimulation. However, if the stimulation is greater, causing nematocyst discharge and tentacle adhesion to the rod, then contraction below the point of attachment occurs, similar to that in the capture of an *Artemia*.

TABLE I

Tentacle movement and position of Artemia *attachment in* H. littoralis

Position of attachment	Number of observations			
	Tentacle movement			Total
	Bending or bending and contracting	Contracting	Spiralling and contracting	
Basal	10	18	0	28
Mid	3	22	3	28
Distal	2	13	15	30
Total	15	53	18	86

(In two *Hydra* the *Artemia* detached and were not engulfed.)

Although the type of tentacle movement depends on the site of prey attachment, the latent period before tentacle movement is independent of the type of tentacle movement. The mean reaction times are not significantly different ($P > 0.10$) among hydra exhibiting the following tentacle movements: (1) bending and contracting (3.2 sec, *Artemia* on basal region); (2) contracting (2.5 sec, *Artemia* on middle tentacle region); and (3) spiralling and contracting (3.0 sec, *Artemia* on distal portion). It was also found that neither the reaction time nor the engulfment time changed significantly ($P > 0.10$) with successive capture for a group of five *Artemia*. Thus, there is no evidence of fatigue or facilitation of movements in the feeding response, at least for the first few prey captured.

2. *Concerted tentacle flexions*

In the majority of hydra investigated, an *Artemia* placed on a single tentacle was engulfed within 80–100 seconds. However, concert activity initiated with the first tentacle movements, continues long after the prey has been swallowed (Fig. 2, upper graph). Only 15–20 minutes after the prey has been engulfed does the concert frequency decrease to the pre-feeding level.

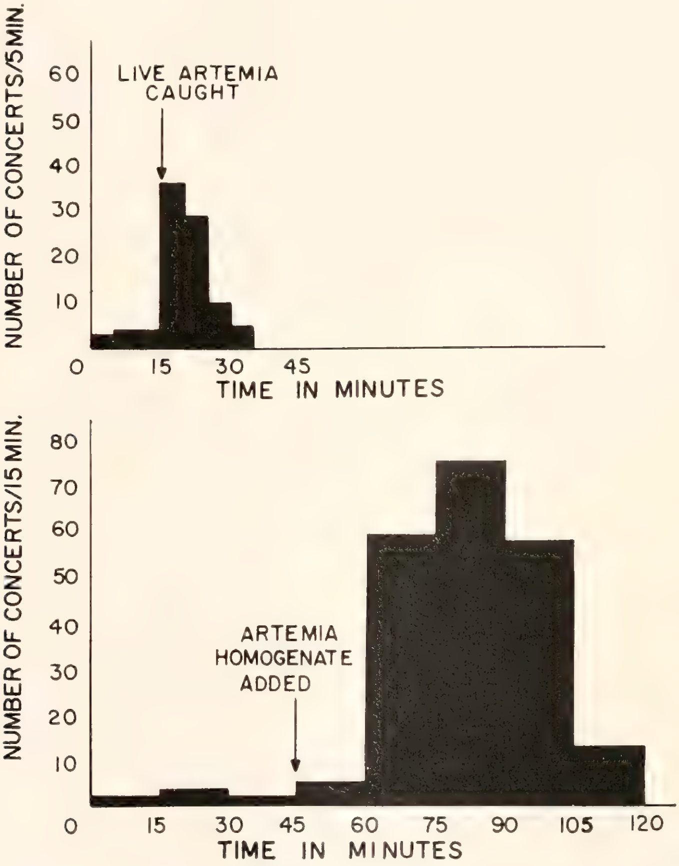

FIGURE 2. Effect of feeding stimuli on concert frequency in *H. littoralis;* upper graph: number of concerts/animal/5 min before and after a single *Artemia* is captured, based on a group of 10 hydra; lower graph: number of concerts/animal/15 min before and after exposure to homogenate of *Artemia,* based on a group of 5 hydra.

Concerted tentacle flexions consist of a highly coordinated oral sweeping movement by all of the tentacles, or all tentacles except that involved in prey capture. Such concerts are quite dissimilar from uncoordinated writhing activities, occasionally seen when the hydra feeds on a single *Artemia.* If several specimens of *Artemia* are simultaneously captured by the polyp, initial concert activity soon gives rise to writhing movements, which continue after all the prey are engulfed.

In turn, the writhing activity becomes less vigorous, and concerts return at enhanced frequencies. Concert frequencies are gradually restored to base line levels, 30–60 minutes later.

Both concerted oral flexion and tentacle writhing can be chemically induced, by either *Artemia* extract or GSH (Fig. 2 and 4). On exposure to *Artemia* extract there are a few initial concerted tentacle flexions, followed by considerable tentacle writhing. Such writhing is the predominant tentacle activity in the first 15 minute period following exposure to the homogenate. Thus, the concert frequency is only slightly above the pre-stimulation level during this period. However, for the subsequent 45 minutes, concert frequency is greatly enhanced, and is still above control values an hour after administration of the extract. This behavioral se-

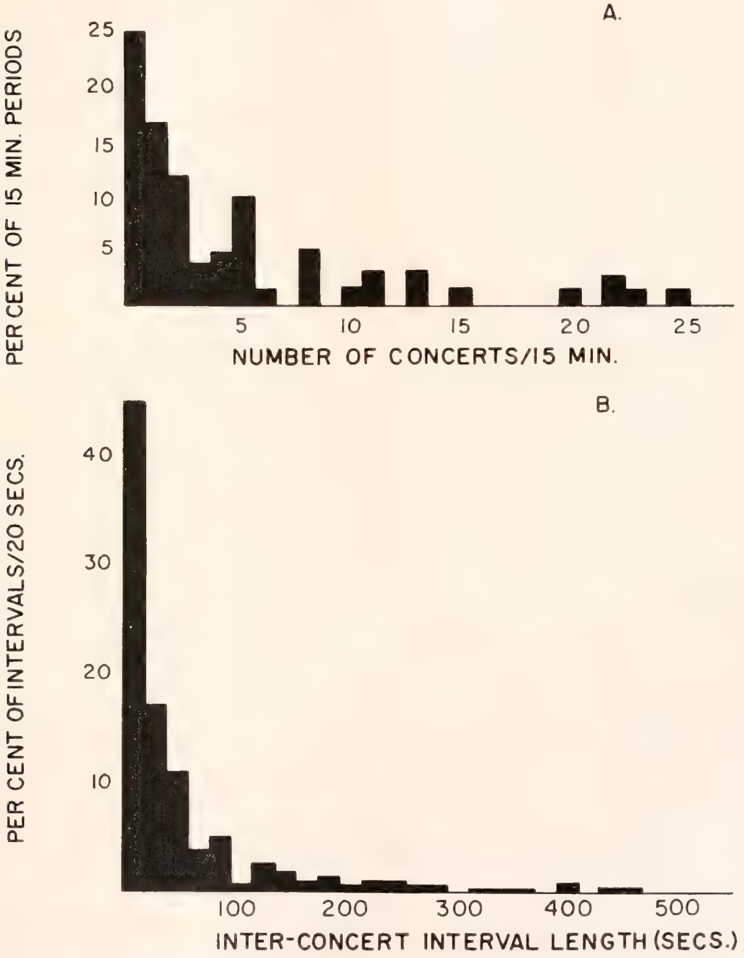

FIGURE 3. Concert frequency in *H. littoralis;* A: per cent distribution of concerts/15 min periods for 60 hydra; B: inter-concert interval distribution for 60 hydra.

quence is also observed when the hydra is exposed to GSH in concentrations greater than 10^{-8} M, or when several live *Artemia* are fed simultaneously to the animal.

A single concert may be induced by prodding a tentacle with a clean glass rod so that nematocyst discharge occurs. The stimulated tentacle adheres to the rod and contracts below the point of attachment, and a concerted flexion of the other tentacles immediately follows. This single concert, therefore, appears to result from mechanical stimulation. Following such stimulation, additional concerts occur at frequencies not greater than prestimulation values, and writhing activity is not initiated.

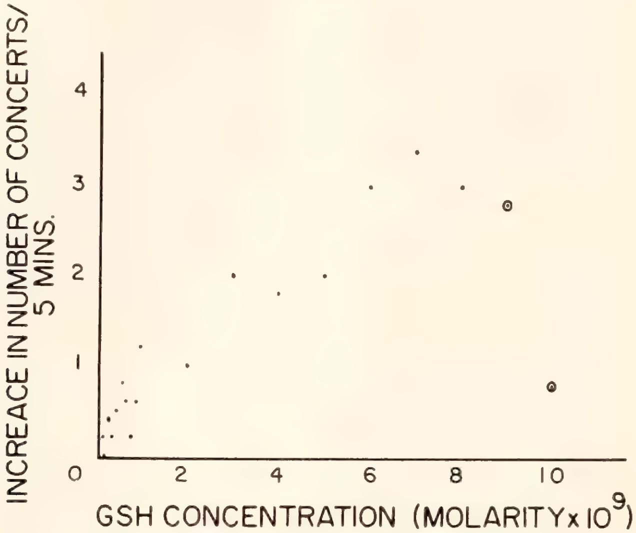

FIGURE 4. Effect of GSH on concert frequency in *H. littoralis;* increase in concerts/animal/5 min as a function of GSH concentration. Each point is based on 50 hydra. The circled values are those readings of concert frequency, in which tentacle writhing accompanied concerts.

Prolonged writhing movements of the tentacles are only seen when the hydra is exposed to chemicals stimulating feeding responses, or noxious substances in high concentrations. This activity is not part of the normal spontaneous behavior of the animal. In contrast, concerts occur endogenously in hydra, their frequency being increased by feeding stimuli. The distribution of spontaneous concerts in unfed *H. littoralis* is shown in Figure 3A. This histogram is highly skewed to the right, a result of the concerts tending to come in bursts. This is clearly seen from the inter-concert interval histogram plotted for these animals in Figure 3B. This distribution is also skewed to the right, with a modal class of intervals having lengths shorter than 20 seconds. These intervals, comprising about 50 per cent of all the inter-concert intervals, represent intervals between concerts in a burst. The longer intervals constitute inter-concert-burst intervals.

In studying the mouth opening response in *H. littoralis* elicited by GSH, Lenhoff postulated a limited number of receptor-effector systems in the animal, localized in the area immediately around the mouth and tentacles (Lenhoff, 1961b). He observed that concentrations of GSH greater than 5×10^{-6} M activate all these systems, 10^{-6} M GSH eliciting a half-maximum response. Experiments with glutathione analogs and related amino acids were used by several workers to document the specificity of glutathione in initiating the feeding response in *Hydra* (Loomis, 1955; Cliffe and Waley, 1958; Lenhoff and Bovaird, 1961). However, both Forrest (1962) and Burnett *et al.* (1963), questioned such specificity, finding that a wide range of substances elicited feeding reactions in several species of *Hydra.* On the other hand, spontaneous column contractions, and those induced by light and mechanical stimulation, were found to be specifically inhibited by GSH and its S-methyl analog (Rushforth, Krohn and Brown, 1964; Rushforth, 1965). It was, therefore, of interest to determine: (1) the degree of specificity of GSH for inducing concerts, and (2) the range of concentrations of GSH over which concert activity was significantly increased.

The following regime was used to assay for GSH and other substances as possible initiators of concerts. The number of concerts were determined for a group of 10 hydra during a five minute period. Then the test substance was pipetted into the dish to give a specified concentration, and the number of concerts/animal was recorded for the subsequent five minute period. The change in the number of concerts between the two time periods was used as a measure of induced concert activity by the substance at the specific concentration. The effect of GSH on concert frequency is shown in Figure 4. There is an increasing rise in concert frequency with higher concentrations of GSH, starting at 1×10^{-10} M GSH levelling off to a plateau value at about 8×10^{-9} M GSH. Although there is considerable variability in concert frequencies, as shown in the wide scatter of points, both the half-maximal and the saturation values are much lower than those for mouth opening or inhibition of contractions, which are in the range of 10^{-6} M GSH (Lenhoff, 1961b; Rushforth, 1965). Thus, this assay shows that extremely small concentrations of GSH increase the frequency of spontaneous concerts. With GSH concentrations above 8×10^{-9} M, concert frequencies decrease as the coordinated sweeping movements of the tentacles change into vigorous writhing activity. Analogs of GSH also increase concert frequency, maximal response levels for S-methyl GSH, S-acetyl GSH and oxidized GSH being attained at concentrations of approximately 8×10^{-8} M, 1×10^{-7} M and 7×10^{-9} M respectively. However, a number of other substances tested at concentrations up to 10^{-6} M had no effect on concert frequencies. These were: proline, glycine, NaCl, NaOH, urea, nicotinic acid, glutamic acid, sodium citrate, and acetic acid, many of which were found by Forrest (1962) and Burnett *et al.* (1963), to elicit feeding activity in *Hydra.*

3. *Inhibition of contractions during feeding*

(a). *Tentacle contractions.* In unstimulated hydra, spontaneous activity of the tentacles consists primarily of single contractions of individual tentacles, or bursts of contractions of one or more tentacles. There is an increased frequency of such tentacle contractions before the contraction of the body column of the hydra (Rushforth, in preparation). During the capture and engulfment of a single *Artemia* endogenous tentacle contractions are supplanted by localized activities of the stimulated tentacle and concerted oral flexions of the rest of the tentacles. With greater stimulation, by multiple *Artemia* or GSH, the tentacle activities consist of uncoordinated writhing movements for extended time periods, gradually giving rise to concerts. During this behavioral sequence, the normal spontaneous contraction activities of the tentacles are suppressed. Those contractions are gradually restored to prestimulation levels, approximately 15 minutes after *Artemia* engulfment (Fig. 5).

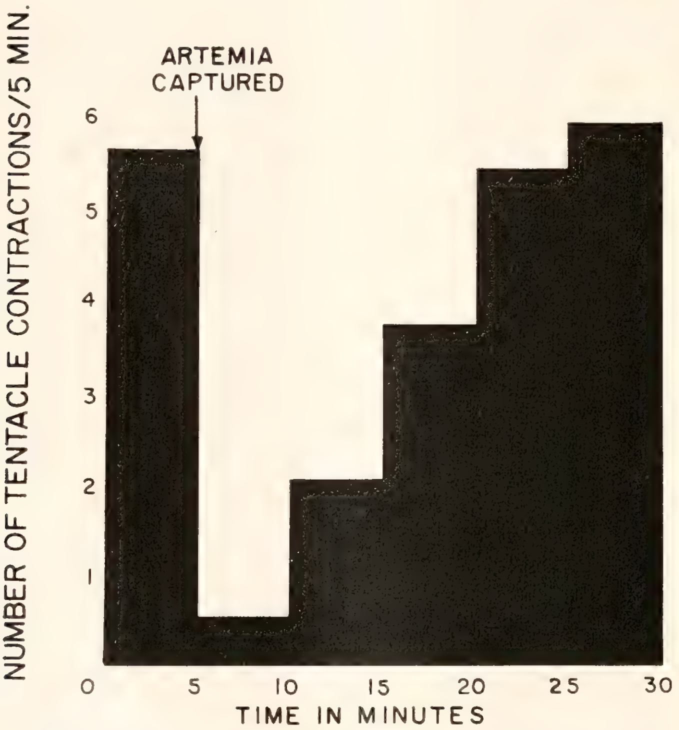

FIGURE 5. Effects of exposure to live *Artemia* on the frequency of tentacle contraction in *H. littoralis;* number of tentacle contractions/animal/5 min, 15 min before and after capture of a single *Artemia,* for a group of 10 hydra.

(b). *Column contractions.* Column contraction is perhaps the most striking spontaneous activity of hydra. In *H. pirardi* most contractions occur in bursts, while in *H. pseudoligactis* contractions tend to occur as single widely-spaced events (Rushforth, 1971). In *H. littoralis* the temporal pattern of contraction activity appears intermediate between that of these two species (Rushforth, 1966). With all species, contractions of the body column are inhibited during feeding (Rushforth *et al.,* 1964). The results of the following experiments clearly show that such inhibition is chemically mediated. One group of 10 hydra was flooded with a large number of *Artemia salina* nauplii and the hydra were allowed to feed for a 15 minute period. If the culture water was not changed at this point, the column contraction frequencies were significantly lower ($P < 0.01$) than those of unfed hydra, over a subsequent 90 minute period (Fig. 6B). If, in contrast, the culture water in which the hydra had been feeding for a 15 minute period was changed, the contraction frequencies were significantly higher ($P < 0.01$) than unfed controls (Fig. 6A). This indicates that the ingestion of *Artemia* itself enhances column contractions while substances liberated to the medium during feeding inhibit them. Inhibition of column contractions occurs when hydra are exposed to: (1) extracts of *Artemia;* or (2) 10^{-5} M GSH (Fig. 6C). Further experiments showed that suppression of column contractions with feeding stimuli occurred with species other than *H. littoralis.* The culture water in which *H. pirardi, H. pseudoligactis* or *H. fusca* had been feeding on excess *Artemia* when *filtered,* significantly reduced column contraction frequencies of *H. pirardi.* In addition, 10^{-5} M GSH inhibited column contractions in all three species.

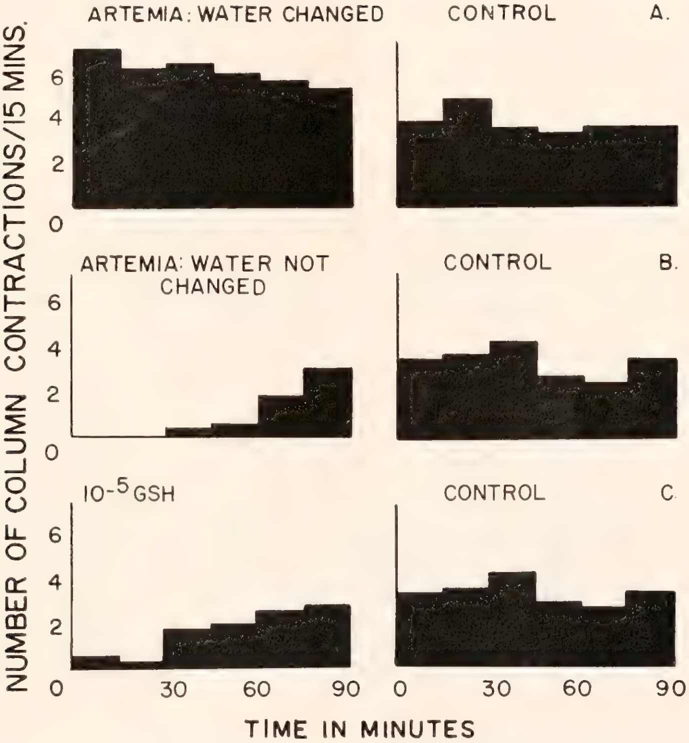

FIGURE 6. Effect of feeding stimuli on column contraction frequency in *H. littoralis;* A: numbers of column contractions/animal/15 min for 10 hydra fed excess *Artemia,* after the culture water was charged (time 0) compared with those for 10 control hydra; B: same as A above when the culture water was not changed; C: column contraction frequency after exposure to 10^{-5} M GSH.

4. Relationship between concerts and tentacle and column contractions

The experiments whose results were described above indicate that feeding stimuli enhance the frequency of concerts while inhibiting tentacle and column contractions. A further experiment was performed to determine the temporal sequence giving rise to increased concert frequency and suppression of tentacle and column contractions when *H. littoralis* was fed a single nauplius. The frequencies of concerts (A), tentacle contractions (B), and column contractions (C), were observed for two control periods of 100 seconds, for each of five hydra. Each animal was given a single *Artemia* and the frequencies of the behavioral events were recorded for the next 30 minutes. The averaged frequencies are plotted in Figure 7. Over the first half of this time period both the frequency of column contractions and tentacle contractions are reduced (Fig. 7B and 7C). A subsequent reduction in concerts appears to be accompanied by a simultaneous restoration of tentacle and column contractions.

The simultaneous increase in concerts and decrease in column and tentacle contractions by feeding stimuli might be explained by one or more of several mechanisms: (1) concerts might be inhibitory to both column and tentacle contractions, such contractions being suppressed as a secondary effect of induced concerts with feeding stimuli; (2) alternatively, mechanisms giving rise to contraction activity may inhibit concerts, and elevated concert frequency may be an indirect effect of suppression of contractions by stimuli eliciting feeding activities; and (3) finally, such feeding stimuli could jointly have direct but opposite effects on concerts and contractions. A series of observations were made to try to distinguish between these possible mechanisms. Unfortunately, these studies were somewhat indirect and the results do not resolve this question.

The frequencies of concerts, column contractions and tentacle contractions were determined for 60 unstimulated hydra of three species for a 15 minute period. The mean frequencies and standard errors, together with correlation coefficients are given in Table II. While *H. littoralis* and *H. pseudoligactis* have similar concert and contraction frequencies, *H. pirardi* has significantly ($P < 0.01$) smaller concert frequency, but greater frequencies of column and tentacle contractions than the other two species. For all three animals, the contraction frequencies for column and tentacles are positively correlated while concert values are negatively correlated with the two types of contractions. Since other studies (Rushforth, in preparation) show that tentacle contractions are most frequent prior to column contractions the negative correlation coefficients suggest inhibition between concerts and contractions.

Passano and McCullough (1964) previously showed that the frequency of contraction bursts of *H. pirardi* decreased with starvation. It was therefore, of interest to determine the relationship between concert activity and the nutritional state of the animal. If concerts were suppressed by tentacle and column contraction, or they were mutually inhibitory, an increase in concerts with starvation, might result from decreased contraction activity. It seemed quite plausible that concert frequency might increase with starvation, since both nematocyst discharge and the duration of mouth opening, two other components of coelenterate feeding behavior are enhanced by starvation (Wagner, 1904; Pantin and Pantin, 1943; Lenhoff, 1961b). However there is a reduction in concert frequency as well as tentacle and column contractions with progressive starvation (Table III).

Excision of the hypostome and tentacles suppresses column contractions in *H. pirardi* (Passano and McCullough, 1964). Removal of the tentacles alone slightly reduces the frequency of spontaneous contraction bursts in this animal (Rushforth, in preparation). If *H. pirardi* is exposed to GSH after removal of the tentacles, the normal inhibitory effects on spontaneous contraction burst activity are observed. Thus, GSH inhibition of column contractions does not take place exclusively by mechanisms requiring input from the tentacle. However, using a more sensitive assay system for the effects of GSH on *H. pirardi*

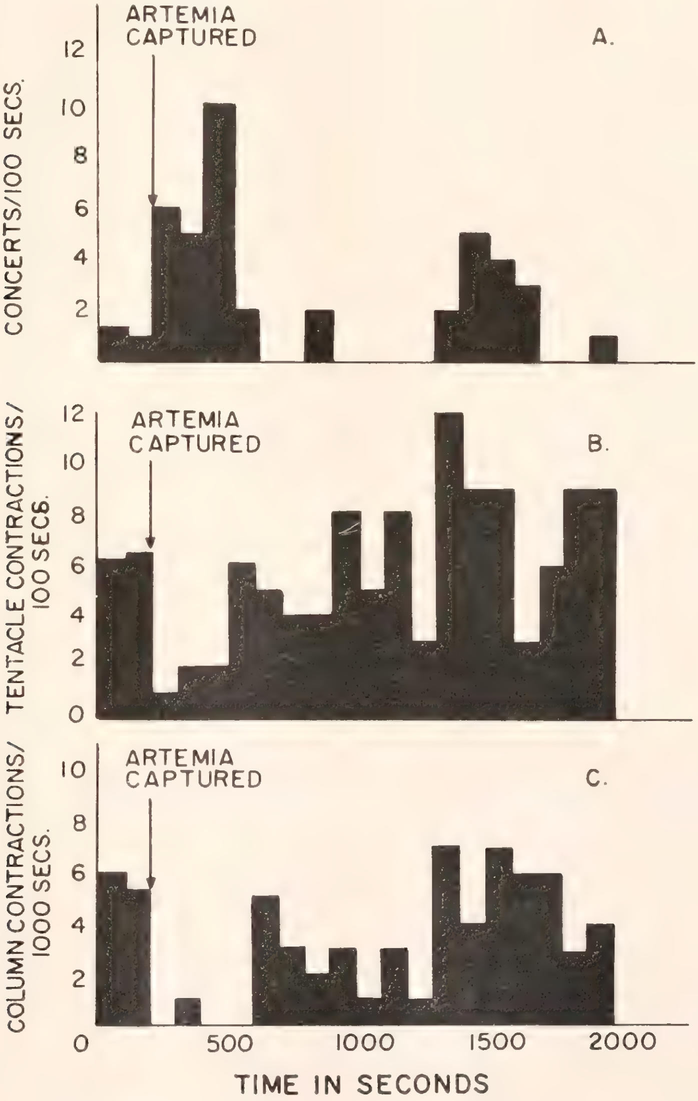

FIGURE 7. Effect of a single *Artemia* on behavioral sequences in *H. littorialis;* A: number of concerts/animal/100 sec before and after *Artemia* capture; B: number of tentacle contractions/animal/100 sec before and after *Artemia* capture; C: number of column contractions/animal/100 sec before and after *Artemia* capture (based on a group of 5 hydra).

TABLE II

Relative frequencies of concerts, column and tentacle contractions in three species of Hydra

	Concerts	Tentacle contractions	Column contractions
H. littoralis	3.1 (0.2)	1.5 (0.2)	4.1 (0.2)
H. pseudoligactis	2.8 (0.3)	1.6 (0.2)	4.4 (0.3)
H. pirardi	0.5 (0.1)	7.9 (0.4)	9.3 (0.4)

	Correlation coefficients		
	Concerts *vs.* tentacle contractions	Concerts *vs.* column contractions	Tentacle contraction *vs.* column contraction
H. littoralis	−0.28*	−0.10	0.29*
H. pseudoligactis	−0.26*	−0.20	0.15
H. pirardi	−0.42**	−0.18	0.53**

* Significant at 0.05 level.
** Significant at 0.01 level.

(Rushforth, 1965), the tentacle structures were shown to play a role in suppressing contractions. *H. pirardi*, with or without tentacles contract to strong light. Removal of the tentacles increases the time between onset of the light stimulation and the initiation of the light-induced contraction burst (Rushforth, in preparation). In addition, the duration of inhibition by 10^{-5} M GSH of light-induced contractions is significantly reduced after tentacle removal (Fig. 8). This result suggests that the tentacles do contribute to GSH suppression of column contractions, and is consistent with Lenhoff's (1961a) hypothesis that GSH receptors are concentrated on these structures.

Electrophysiological correlates of feeding responses

Passano and McCullough (1963) showed that both tentacle and column contractions were preceded by large compound potentials. Electrical recordings of individual hydra were taken before, during and after exposure to feeding stimuli, in order to determine if there were electrical correlates for any of the behavioral modifications previously described. Joint observations of the behavior and electrical

TABLE III

Effects of starvation on the frequencies of concerts, column and tentacle contractions in H. littoralis
Number/15 minutes

Days since feeding	Concerts	Tentacle contractions	Column contractions
0	3.8	1.0	3.5
1	2.0	1.0	3.0
2	1.3	0.5	0.8
3	1.2	0.5	1.2
4	0.5	0.2	0.8
5	0.5	0.2	0.8

activity of several species of *Hydra* have failed to show electrical potentials associated with spontaneous concerted oral flexions. However, Tentacle Contraction Pulses (TCP's) and Column Contraction Pulses (CP's) always associated with tentacle contractions and column contractions respectively, occur spontaneously as single events and in bursts. When *H. pirardi* feeds on *Artemia,* CP bursts and singles are suppressed. TCP bursts are absent although single TCP's occur during this period. Rhythmic pulses are evident throughout feeding, as was first observed by Passano and McCullough (1963). After ingestion of the *Artemia,* CP's return first as single events and then as CP bursts of enhanced frequency. Associated with the return of CP's is a marked increase in TCP firing.

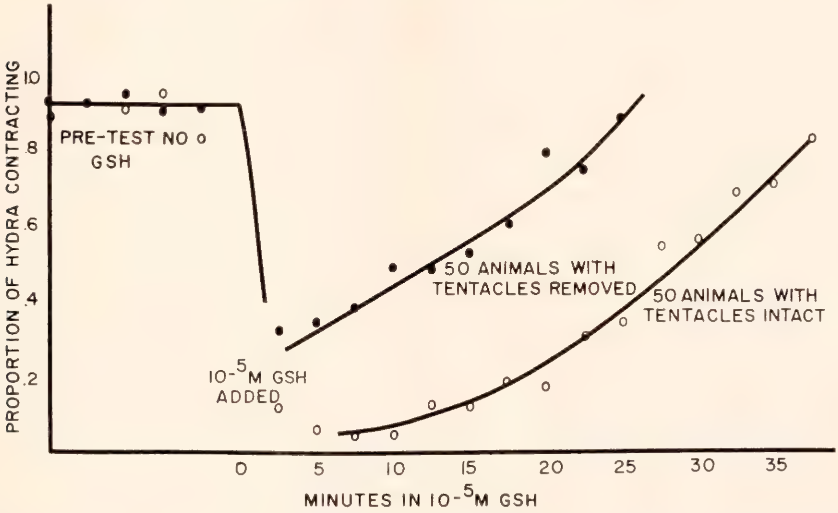

FIGURE 8. Effect of tentacle removal on light-induced contractions in *H. pirardi.* Per cent of a group of 50 hydra with tentacles removed (closed circles) and a group of 50 intact hydra (open circles) contracting in a 75 second period of bright light followed by a 75 second period of ambient illumination.

When a preparation of *H. pirardi* is exposed to 10^{-5} M reduced glutathione, effects similar to those resulting from contact with *Artemia salina* occur in pacemaker activity. Column Contraction Pulses are inhibited, returning first as singles and then as CP bursts. Tentacle contraction bursts are also inhibited but return as TCP bursts. The Rhythmic Potential Pacemaker System is not, however inhibited by GSH. The frequencies of TCP's and CP's are markedly reduced on administration of GSH, but return at higher than prestimulation values, before returning to control levels. As in the case of exposure to *Artemia,* inhibition of CP's is longer than for TCP's. The duration of the blockage of CP's in *H. littoralis* was determined as a function of GSH concentration over the range 10^{-5} to 10^{-9} molar. The length of inhibition of spontaneous CP's increases with GSH concentration from 10^{-9} to 10^{-7} M (Fig. 9).

In the final stages of formation, prior to detachment from the column of the hydra, a bud exhibits CP's independently of the parent's CP's. However, if feeding stimuli are administered to the preparation, both parent and bud CP's are suppressed. This preparation afforded a suitable method of showing that during a capture of an *Artemia* by the parent hydra sufficient chemical stimulation is released to suppress CP's of the bud. The same effect was observed using two budless specimens of *H. littoralis*. The capture of *Artemia* in one hydra released sufficient chemical feeding stimuli not only to suppress CP's of that animal, but also to inhibit CP's of the other hydra for a short time period. In addition, the concert frequencies were significantly increased in both animals.

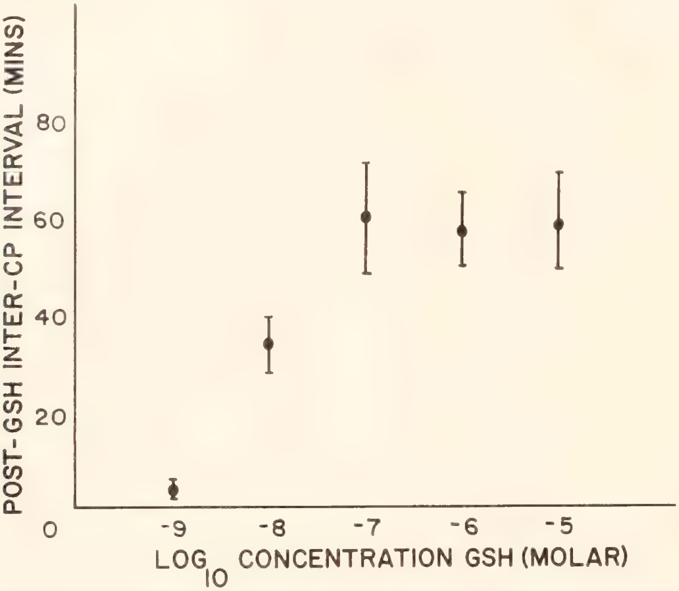

FIGURE 9. Effect of GSH concentration on inhibition of CP's in *H. littoralis*, Post-GSH inter-CP interval plotted against $\log_{10}$ GSH concentration. This interval length measures the time in minutes from a CP immediately preceding GSH administration to the next CP that is followed by another CP within a 10 minute period. Mean values and ranges are based on five hydra.

DISCUSSION

1. *Overt behavioral responses of feeding*

As previously pointed out by Josephson (1965c, page 34) the mechanisms underlying feeding behavior in *Hydra* can be explained as "linked sequences of local responses, each component being initiated by the results of the preceding one." The first element in feeding behavior is the attachment of the prey to an outstretched tentacle by means of nematocyst discharge. The two types of nematocyst involved in prey capture, stenoteles and desmonemes, both discharge to the mechanical stimulation caused by prodding the tentacles with a clean glass rod. The threshold for such activation is drastically lowered, however, by the presence of food extracts. This has been shown by Pantin (1942) for the anemone *Anemonia*, and for *Hydra* by Ewer (1947). The desmonemes coil around the prey entrapping it, while the stenoteles pierce its exoskeleton in a harpoon-like fashion. The discharged nematocysts are released from the tentacle, as the prey organism is enclosed by the mouth (Ewer, 1947).

As the prey is attached to the tentacle by nematocyst discharge, after a latent period of a few seconds, the stimulated tentacle moves to bring the prey in contact with the mouth. Josephson (1965c) observed that sometimes this latency lasts up to a minute, while in the hydroid *Pennaria* a delay lasting up to 30 seconds is not uncommon (Fred Delcomyn, University of Oregon, personal communication). Such latencies appear surprisingly long and are difficult to explain. Possibly they occur in cases where the *Artemia* is ensnared so that the initial movements of the prey are not vigorous enough to elicit immediate tentacle movement. However, as the *Artemia* continues to struggle, finally the threshold for tentacle activity is reached.

Distinctly different tentacle movements are produced depending on the position of prey attachment. However, the reaction time appears independent of the type of movement. This result strongly suggests that the times for conduction of impulses, or diffusion of chemical factors from the point of prey attachment to the hypostome, where presumably coordination of tentacle movements takes place, do not play a major role in determining the reaction time. The occurrence of such movements following nematocyst discharge with mechanical stimulation by an inert object, suggests that the stimulus from the prey for these behavioral components is probably mechanical in nature. The contraction of the tentacle below the point of prey attachment, and not above it, implies polarized conduction towards the hypostome.

A third behavioral sequence in the feeding response, concerted oral movements of the tentacles, is primarily a result of chemical stimulation. While such concerts occur spontaneously, usually in bursts, their frequency is increased by dilute extracts of *Artemia* or extremely small concentrations of reduced glutathione or its analogs, (*i.e.,* below 10^{-8} M). Such chemical stimulation may occur in nature by release of GSH from the prey organism, as was proposed for other elements of feeding behavior by Loomis (1955). Alternatively, the release might be from the discharged nematocyst capsule itself as postulated by Burnett *et al.* (1963), although Lenhoff (1968a) has shown this second alternative to be improbable in the case of the mouth opening response (which occurs at a much higher GSH concentration). Chemical activation of repeated concerts at dilute concentrations, and of tentacle writhing at higher concentrations, results from stimulation via the external environment. However, a single concerted oral flexion of the tentacles invariably follows movement of a stimulated tentacle if nematocyst discharge occurs with mechanical stimulation. This suggests that the concert and basal tentacle contraction may be linked internally rather than through the external medium. Tentacle writhing and mouth opening are chemically initiated (Wagner, 1904; Ewer, 1947; Loomis, 1955; Lenhoff, 1961b). Both sequences are induced by GSH at concentrations ($10^{-6} - 10^{-7}$ M) much higher than that giving maximal concert frequencies in *H. littoralis* (about 10^{-8} M). The lower threshold for chemical stimulation of concerts than for mouth opening, suggests why concerts occur earlier in the feeding response. Concerted oral flexions of the tentacles occur when the prey organism is held above the hypostome before ingestion. Frequently the tentacle opposite to that attached to the *Artemia* pushes the prey towards the mouth region in the concert movement. At higher GSH concentrations the mouth begins to open and creep over the prey, and the concerts give rise to uncoordinated tentacle writhing. Possibly such writhing movements are the result of excess stimulation of mechanisms coordinating the concert activity.

The later stages of feeding behavior in *Hydra* consist of mouth creeping around the prey, closing about it, a reduction in the vigor of tentacle writhing, and a gradual return of coordinated concert frequencies to base-line levels. Throughout the feeding sequences the endogenous contractions of both the tentacles and column are inhibited. They return after *Artemia* ingestion, at enhanced frequencies, before they are restored to prestimulation values. Inhibition of contractions and their associated electrical potentials will be discussed later.

Little is known concerning the mechanisms involved in mouth closing and creeping about the prey. Creeping of the mouth may result from stimuli due to contact of the prey on the inside of the mouth. An inert object, such as a pin, will be swallowed when it is placed in the mouth of hydra, in the absence of added GSH (quoted in Kanaev, 1952). An isolated hypostome will creep slowly up and off the shaft of a pin inserted through the mouth (Rushforth, unpublished observations). The hypostome always moves in an oral direction, at a reduced speed if the tentacles are removed. The present day description of mouth movements in the feeding response of *Hydra* go little further than those of Hartog (1880), given almost a century ago. He pointed out that the tentacles play little role in the feeding response as soon as the mouth comes into contact with the food.

Very little is known of the mechanism responsible for mouth closure and termination of the various behavioral sequences. Burnett, Lentz and Warren (1959) show that fully fed hydra are capable of discharging nematocysts and subduing prey. However, once the gastrovascular cavity of the animal is filled, the hydra does not attempt to ingest the captured prey. The mouth appears incapable of opening, due to the internal pressure exerted on the walls of the gastrovascular cavity, as a result of a large intake of water during the early phases of digestion. However, during an extended period following ingestion of *Artemia* (1–6 hours) the mouth may open to engulf single prey while the hydra forms a tight constriction in the region just below the hypostome (Blanquet and Lenhoff, 1968). This "neck formation" was shown in *H. pirardi* to be caused by a combination of three factors: (a) glutathione on the exterior of the hydra; (b) distension of the wall of the hydra's body column; and (c) the presence of tyrosine within the gut. Tyrosine was found to be highly specific in causing neck formation. No other amino acid, including phenylalanine was active, and tyrosine having either the α-amino or α-carboxyl blocked had no effect (Blanquet and Lenhoff, 1968).

2. *Electrical correlates of feeding behavior*

Throughout the initial phases of feeding the spontaneous contraction of the tentacles and body column are suppressed. These recurring simple behavioral events which result from pacemaker activity, are supplanted by the linked sequences of local responses described above. Similar effects are seen with other coelenterates. Long term modification of the rhythmic contractions of the body wall of *Metridium* have been observed with food extracts (Pantin, 1950). Suppression of the swimming response by food extracts was also reported by Ross and Sutton (1964) for *Stomphia*. The control mechanisms involved in swimming and feeding in this sea anemone appear mutually inhibitory, since there is also suppression of nematocyst discharge during swimming. Such findings suggest that nematocysts do not always act as purely independent effectors. Inhibition of the discharge of atrichous isorhizas, the nematocyst type used in hydra's locomotion has been found to occur with food extracts (Ewer, 1947).

In *Hydra,* feeding stimuli also inhibit the production of electrical potentials which are associated with column contractions (CP's) and contractions of the tentacles (TCP's). After ingestion of *Artemia* both the contractions and their associated potentials return at enhanced frequencies. It was initially thought that the increase of body contractions resulted from the mechanical stimulation to the gut of the hydra produced by the engulfed *Artemia.* However, the same effect was observed with extracts of *Artemia* and 10^{-5} M GSH. The process is reminiscent of post-inhibitory rebound observed in neural systems (Erlanger and Glasser, 1937) and occurs in *Hydra* whenever the endogenous contractions and correlated bioelectric events are inhibited by external stimuli (Passano and McCullough, 1964; Rushforth, 1971).

In another hydroid, *Tubularia,* concerts and associated electrical potentials are suppressed during feeding (Josephson and Mackie, 1965), and are inhibited by the presence of food extracts (Rushforth, 1969). In view of the inhibitory effects on both the behavior and electrical activity of *Hydra* and *Tubularia,* together with the reports of suppression of behavioral events with feeding in sea anemones, one may conjecture as to the generality of the phenomenon. Clearly even in other hydroids, spontaneously recurring behavioral events and large compound electrical potentials are not always present. However, in *Cordylophora, Pennaria* and *Hydractinia* where there is an apparent lack of endogenous behavior and electrical activity, feeding leads to a dramatic electrical awakening in such polyps (Mackie, 1968, Fred Delcomyn, personal communication). As a working hypothesis, it is proposed that in hydroids and possibly other coelenterates, feeding stimuli, in addition to initiating reflexly linked local responses either (1) inhibit spontaneous movements and correlated potentials or (2) induce electrical activity in normally quiescent animals. In addition, such feeding stimuli may inhibit responses to external stimuli: light and mechanically induced contraction in *Hydra* (Rushforth, 1965); swimming responses in *Stomphia* initiated by inert objects previously rubbed on the aboral surface of the starfish *Demasteras* (Ross and Sutton, 1964); and gastrozooid contraction following a strong mechanical stimulus to the basal mat of the colony of *Hydractinia* (Darrell Stokes, personal communication).

The mechanisms by which feeding stimuli simultaneously elicit concert activity, and suppress tentacle and column contractions in *Hydra,* are unknown. Sustained behavioral observations together with electrical recordings have failed to reveal electrical correlates of concert activity. Even in recordings of isolated tentacles no pulses were observed to correspond with sweeping movements presumably the same as concert activity in the whole animal. In this preparation, when tentacle writhing and asymmetric movements occurred in response to feeding stimuli, characteristic single monophasic potentials supplant the normal bursts of pulses (TCP's) associated with symmetric contractions of the longitudinal muscles (Rushforth and Burke, 1971). However, there is insufficient evidence to indicate that the monophasic pulses are produced by a discrete pacemaker system separate from the Tentacle Contraction Pacemaker System.

The mode of inhibition of column contractions and the associated CP's is also unknown. It is unlikely to result from direct effects on the transepithelial potential (Josephson and Macklin, 1969) since GSH while drastically modifying CP activity has no apparent effect on this potential (Rushforth and Burke, in preparation). In *Tubularia*, inhibition of concerts and associated HP's with live *Artemia* or *Artemia* extracts occurs by activating a specific conducting system, the Distal Opening System (Rushforth, 1969). This system produces small potentials (DOSP's) on electrical stimulation, and was previously shown to be inhibitory to pacemaker systems, and to suppress concerts, as it initiated aboral flaring of the distal tentacles (Josephson and Uhrich, 1969). Possibly in *Hydra* a similar mechanism occurs, but no conducting system similar to the Distal Opening System has been discovered. It should be recalled that in *Hydra* not all of the pacemaker systems are inhibited during feeding, since RP's occur throughout the sequences of the feeding behavior (Passano and McCullough, 1965). In *Sarsia*, feeding produces "swallowing pulses" which are conducted from the manubrium to the tentacle bases; these pulses seem to inhibit swimming pulses by the latter's pacemakers (Passano and Hernandez-Nicaise, 1967).

In the past decade considerable effort has been invested in studies of the "physiochemical aspects of the macro- and micro-environments surrounding *Hydra* during activation of their feeding behavior" (Lenhoff, 1965). However, our knowledge of mechanisms within the animal, controlling its sequential feeding responses is quite rudimentary. Clearly both excitatory and inhibitory processes are involved, and sequences appear to consist mainly of local responses, linked by stimuli from the external environment. In some coelenterates coordination of contiguous effectors via externally mediated stimuli may be associated with inhibition of pacemaker activity. The possible interrelationships between the two types of behavior nevertheless provide challenges for future study.

SUMMARY

The capture and engulfment of a single *Artemia* nauplius by *Hydra* consists of a series of complex behavioral sequences: (1) nematocyst discharge; (2) tentacular movements; (3) mouth opening, creeping over the prey and closure; and (4) inhibition of endogenous tentacle and body column contractions. The present study focused on two of these sequences: tentacular movements and inhibition of tentacle and column contractions.

On attachment of the prey to a tentacle by nematocysts, there is a latent period (1 3 sec in *H. littoralis*). Then the portion of the tentacle proximal to the prey contracts, sometimes accompanied by oral bending or inward spiralling when the prey attaches to the basal or distal tentacle regions, respectively. The latency is independent of the position of attachment or the type of tentacle movement. This

implies that neither conduction time nor the time for the diffusion of chemical factors to the hypostome is a predominant component of the latent period.

As the prey nears the mouth on tentacle contraction, the surrounding tentacles concertedly flex orally (a concert). Concerts are frequent during and following the engulfment of prey. They are highly coordinated movements, unlike tentacle writhing, which is also frequently observed when hydra feeds. Concert frequency is markedly enhanced by exposure of the hydra to homogenates of *Artemia,* reduced glutathione (GSH) or analogs of GSH. In *H. littoralis* concert frequency increases with GSH concentration starting at 1×10^{-10} M GSH and levelling off to a plateau value at about 8×10^{-9} M GSH, above this concentration tentacle writhing is induced. No electrical correlates of concerts have been observed.

Column and tentacle contractions are inhibited during feeding. Such inhibition is also observed with extracts of *Artemia* and GSH. Concerts are negatively correlated both with tentacle and column contractions in several species of *Hydra.* However, the spontaneous frequencies of all three behavioral events decrease with starvation over a period of several days. GSH inhibits endogenous column contractions in *H. pirardi* without tentacles, but removal of tentacles significantly reduces inhibition of light-induced contractions in this animal.

Electrical potentials associated with tentacle contractions (TCP's) and column contractions (CP's) are suppressed when hydra feed on *Artemia* or are exposed to extracts of *Artemia,* or GSH. After such inhibition both types of potentials return together with contractions, at enhanced frequencies. The duration of the blockage of spontaneous CP's increases with GSH concentration over the range 1×10^{-9} M to 10^{-7} M, above which the length of inhibition is constant. A single *Artemia* fed to a hydra releases sufficient chemical factors into the medium to increase concert frequencies and suppress CP's in an attached bud of the fed animal, or in a second hydra some centimeters away from it.

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STUDIES ON THE DEVELOPMENT OF THE SEA URCHIN *STRONGYLOCENTROTUS DROEBACHIENSIS*. I. ECOLOGY AND NORMAL DEVELOPMENT

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The sea urchin *Strongylocentrotus droebachiensis* is one of the most widely distributed of the echinoderms. Its occurrence is circumpolar, extending into the boreal regions of both the northern Atlantic and Pacific Oceans (Mortensen, 1943). This organism was the first echinoid to be described embryologically (Agassiz, 1864) but identity of later larval stages was a matter of dispute for some time (Mortensen, 1943). The true echinoplutei were finally described over a century after Agassiz's original study (Strathmann, 1971).

Ethel Browne Harvey, in her classic work "The American Arbacia and Other Sea Urchins" (Harvey, 1956), treats this species only briefly. Both Harvey (1956) and Boolootian (1966) describe a comparatively short breeding season for specimens obtained north of Cape Cod. The fertilized eggs typically develop with asynchrony, are large and yolky, and have heavy jelly coats. These points have caused *S. droebachiensis* to be regarded as a rather undesirable organism for the more sophisticated studies of cell division and early development. Some large-scale biochemical work has recently been carried out with gametes of *S. droebachiensis* (*e.g.*, Kolodny and Roslansky, 1966; Stephens, 1967; or Stephens, 1970) but the "more reliable" *S. purpuratus* or *Arbacia punctulata* dominate the literature of cellular and developmental biology.

Neither Agassiz's early account (1864) nor any later work describes the development of *S. droebachiensis* under controlled laboratory conditions. This report presents such sequences, relating development to natural environmental temperatures, and consequently suggests optimal conditions for fertilization and synchronous development. These methods and developmental schemes serve as the basis for further studies on mitotic spindle assembly and control, patterns of protein synthesis, and the mechanism of ciliogenesis (Stephens, 1972; in preparation).

MATERIALS AND METHODS

Experimental animals

Strongylocentrotus droebachiensis, 2″–4″ in diameter, was collected from various subtidal areas of Cape Cod Bay during the fall and winter of 1966–1971 and maintained either in running sea water of ambient ocean temperature or in closed aquaria at 4° C utilizing sub-sand filtration. The food supply for animals maintained in running sea water consisted of *Laminaria* while that for animals in the closed system consisted of a mixed algal population growing on the walls of the tank; the latter animals were generally used within a week or two after collection. In the Cape Cod population, no differences in the degree of gonad development were noted between freshly-collected specimens and those maintained in the running sea water system. This might be expected since both sets of specimens experienced essentially the same seasonal temperature change and had available the same food supply. Ripe animals maintained in the closed system at 4° C remained in breeding condition for at least two months beyond the time of natural spawn-out, if fed an occasional piece of *Laminaria*. For comparison, urchins were also obtained from the Boothbay Harbor area of Maine through commercial sources and were generally used immediately. Urchins collected from Maine prior to the breeding season and maintained in the Woods Hole sea water system underwent gonad development coincident with those of the native Cape Cod population.

Gametes

Eggs were obtained by injection of 0.53 M KCl into the perivisceral cavity (Palmer, 1937; Costello, Davidson, Eggers, Fox, and Henley, 1957). For a typical 3″ diameter urchin, two injections of 2 ml each were made at diametrically-opposite points in the peristome. The urchin was placed atop a 100 ml beaker filled with filtered, ice-cold sea water, aboral side down, and the beaker nearly immersed in cold running sea water. Thirty minutes were usually sufficient to obtain the maximum number of eggs, even at 0° C. Alternatively, eggs may be shed in a refrigerator or cold room whose temperature does not exceed 8° C.

Sperm were obtained by removal of the testes when a male was detected by the above injection method. The testes were briefly rinsed in cold sea water, lightly blotted, and placed in a covered plastic petri dish on ice. "Dry sperm," exuded from the testis, was diluted 1:20 with cold sea water containing 10^{-4} M EDTA (Tyler, 1953) and used for fertilization within 5 minutes.

After shedding, the eggs were washed by decantation at least twice with 10 times their volume of cold filtered sea water. The eggs were either used immediately or else washed once more with cold Millepore-sterilized sea water containing 0.05% sulfadiazine (Tyler and Tyler, 1966), resuspended in fresh cold sulfadiazine-sea water, and kept on ice in a covered Stender dish. The eggs were adjusted to such a concentration that they formed a layer no more than two cells thick; the depth of the fluid in the dish was 1 centimeter. Under these conditions, 90–95% of the eggs were fertilizable for 1–2 days; thereafter fertilizability dropped off to about 10% in 7 days. Eggs fertilized during this 1–2 day period developed normally but not nearly as synchronously as those fertilized immediately after shedding.

Fertilization and development

One ml of 1:20 sperm suspension was typically mixed with 10 ml of eggs in 200 ml of sea water. The eggs were allowed to settle, the sea water was decanted, and the eggs were washed twice with filtered sea water. The egg suspension was partitioned into Stender dishes in such a manner that the eggs formed a layer no more than two cells thick, in fluid no more than 1 cm in depth.

The dishes were then maintained on ice, in the running sea water system, or in a temperature-controlled water bath. The fertilization, washing, and transfer operations were carried out at the same temperature as embryonic development.

Removal of the jelly coat

The developmental studies carried out in this report were all done with untreated eggs, but often removal of the jelly coat (Tyler and Tyler, 1966) is sometimes desirable in order to decrease the egg volume, to remove adhering bacteria, or for various biochemical isolation procedures. The eggs, in at least 10 times their volume of cold sea water, are rapidly adjusted to pH 5 (no less!) with dropwise addition of 0.1 HCl. The eggs are immediately spun down in a hand centrifuge and washed three times by centrifugation with cold filtered sea water. Exposure to acid conditions must be minimized in order to obtain maximum fertilization and optimum synchrony. When this procedure is carried out carefully, there are no significant developmental differences between normal and de-jellied eggs.

Glassware

All glassware used was soaked for at least one hour in Alconox detergent, rinsed 10 times with tap water, 10 times with deionized distilled water, and allowed to dry protected from dust or vapors of fixatives. Containers for handling the eggs or embryos were pre-chilled to the temperature of the experiment. Sterile disposable plastic petri or tissue culture dishes (Fisher or Falcon) were found to be non-toxic for both fertilization and development, requiring no pre-washing.

Microscopy

Developing embryos were photographed through either Zeiss phase-contrast or Leitz polarization optics. Fields of embryos were photographed through a Wild M-5 stereomicroscope equipped with a phototube and Polaroid filters. Ciliary motility in later stages was arrested with osmium tetroxide vapor fixation. Flattening of embryos was prevented through the use of 0.18 mm thick coverglass placed beneath the usual coverglass as a spacer. Kodak Panatomic-X film was used throughout and was developed in Kodak Microdol-X developer. Magnification calibration was obtained through photographs of a stage micrometer taken through the same optical system as the embryo.

RESULTS

Breeding season

Based upon observations made since the winter of 1966–67, the breeding season of *S. droebachiensis* from Cape Cod Bay encompasses nearly four months. Ripe eggs can be obtained in mid-December from about $\frac{1}{10}$ of the females and by late January nearly half of the females are fertile. The period from mid-February until mid-April represents the maximum period of fertility with 99% fertilization in $\frac{9}{10}$ of the females; maximum egg volume and essentially 100% fertilization are found from mid-March until mid-April, after which a rapid spawn-out takes place.

During particularly cold winters, the fertility period extends into May. The males are ripe nearly two months earlier and remain ripe about a month later than the females; sperm can usually be obtained throughout the year in small amount. No attempt was made to relate gonad index (weight of gonad/weight of animal) to fertility for it was found that the gonad index in early January was not significantly different from that in mid-April, the former time representing gonads full of eggs but in the germinal vesicle stage.

Urchins obtained from Maine in 1964–65 and 1966–67 showed a much more limited season, with the beginning of the season taking place about one month later and spawn-out occurring coincident with or even two weeks earlier than in urchins from Cape Cod Bay. Figure 1 illustrates monthly mean water temperatures off Boothbay Harbor, Maine, and Woods Hole, Massachusetts; superimposed upon these temperature curves are data relating to the breeding season of *S. droebachiensis* from Maine and from Cape Cod Bay.

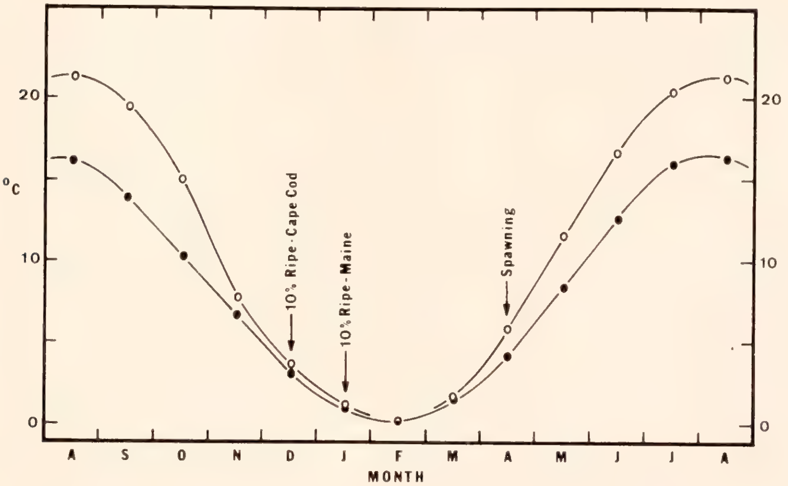


FIGURE 1. Mean monthly sea temperature for Boothbay Harbor, Maine (filled circles) and for Woods Hole, Massachusetts (open circles); from the data of Taylor, Bigelow, and Graham, 1957.

Some qualitative differences in egg properties have been noticed in animals collected from these two populations, but these differences are difficult to evaluate. Most batches of eggs from the northern *S. droebachiensis* are yellow-orange in color and are frequently mixed with large amounts of mucus. The urchins from Cape Cod Bay also produce some batches of eggs with this intense coloration but most are yellow or even pale-yellow to colorless; they are consistently free of mucus. Taken at the maximum of the breeding season, eggs from the northern population are somewhat less synchronous than those from Cape Cod Bay. Maine urchins wholely ripened in the running sea water system at Woods Hole show no such differences in color or synchrony, so such effects are probably environmental, most likely related to food supply.

Developmental sequence

Even though having twice the diameter and developing at temperatures 10–20° C lower, the fertilized egg of *S. droebachiensis* develops along a time scale proportional in all respects to that of the more commonly studied *Arbacia.* Table I lists events in the first division of *S. droebachiensis* at 0° C, 4° C, and 8° C. When temperature variation is held to within ± 0.2° C, synchrony at the first division is excellent. At 8° C, 90% of the cells cleave at ± 5 minutes of the time cited. At 0° C, the comparable range is ± 15 minutes. In both cases, this range represents an interval of about 6% of the total division time. With temperature fluctuations of 1–2° C, particularly at prophase, the degree of asynchrony is doubled or tripled. Figure 2 plots the log of the time of metaphase and cytokinesis *versus* temperature for both the first and second division of *S. droebachiensis.* Temperatures above 10° C cause gross asynchrony while those above 12° C arrest cell division irreversibly in at least 80% of the cells; 14–15° C is lethal.

TABLE I

Temperature dependence of first division events, time in minutes (± 5%)

	0° C	4° C	8° C
Fertilization membrane complete	10	7	5
Sperm aster	80	50	30
Union of pronuclei	110	65	40
Hyaline layer complete	200	120	75
Nuclear membrane breakdown	350	210	130
Metaphase	405	250	150
Anaphase	430	260	160
Telophase	460	280	170
Cleavage	510	310	190

Fry (1936) has noted that, in *Arbacia,* metaphase always occurs at a time point that is 80–85% of the cleavage time regardless of temperature; R. E. Kane of the University of Hawaii (personal communication) has made similar observations with regard to the various temperate and tropical sea urchins that he has studied. It is obvious from Figure 2 that this rule holds true in the case of *S. droebachiensis* over its entire temperature range in both the first and second division.

The developmental stages through the four-armed echinopluteus (whereafter feeding is necessary) at 8° C are illustrated in Figure 3, demonstrating both the size and the relative clarity of the egg and embryo. Table II contains additional information about later events in development at 0° C, 4° C, and 8° C.

Regardless of temperature, problems occur at the hatching of the blastula. Growth in sea water from the laboratory system is quite normal up to this point, but after hatching the blastomeres disintegrate. Transfer to Millepore-sterilized sea water or sea water containing sulfadiazine just prior to hatching entirely prevents this disintegration. Tyler and Rothschild (1951) recommend penicillin for apparently the same reason. Other marine embryos (*e.g., Arbacia punctulata* or

Asterias forbesi) show no such sensitivity when grown in untreated sea water. Examination of embryo cultures after disintegration reveals a large population of *Pseudomonas* sp. and other unidentified bacteria. Apparently *S. droebachiensis* is particularly susceptible to bacterial action.

DISCUSSION

Conditions influencing breeding

Harvey (1956) reports that *S. droebachiensis* from Salisbury Cove, Maine, had unripe eggs in January and February, showed maximum ripeness in March and April, and was fully spent by mid-May. Boolootian (1966), on the other hand, cites February and March as the peak season, with some ripe gametes obtainable in January but spawn-out in March for animals collected from Lamoine, Maine. The animals employed in this report, obtained from Boothbay Harbor, Maine, correspond most closely with Harvey's data, while the Cape Cod Bay population appears to ripen coincidentally with the population cited by Boolootian but remains ripe much longer. Sverdrup, Johnson, and Flemming (1942) report that *S. droebachiensis* in Norway has a breeding season from December through April, but gives no location, quantitative fertility data, nor seasonal temperature variation.

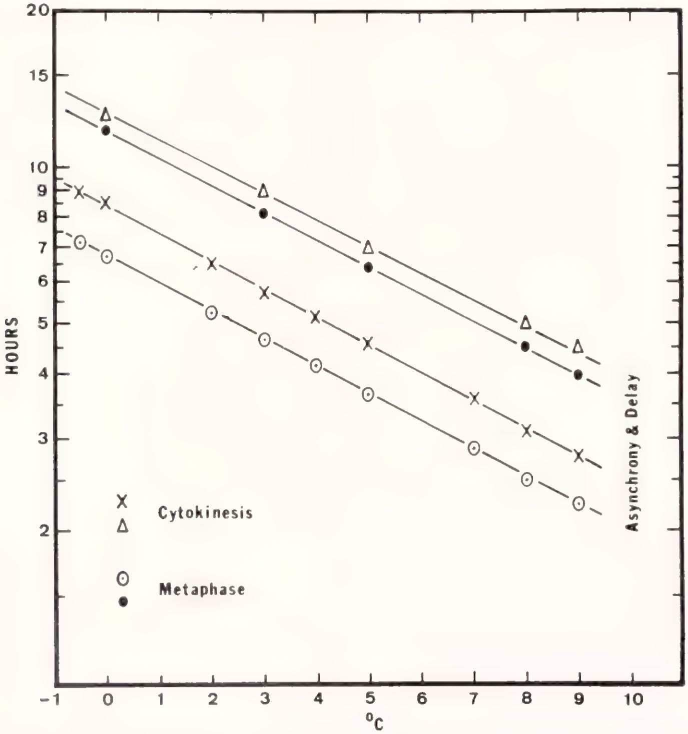


FIGURE 2. Semi-logarithmic plot of the mean time for metaphase and cytokinesis *versus* temperature for the first and second divisions.

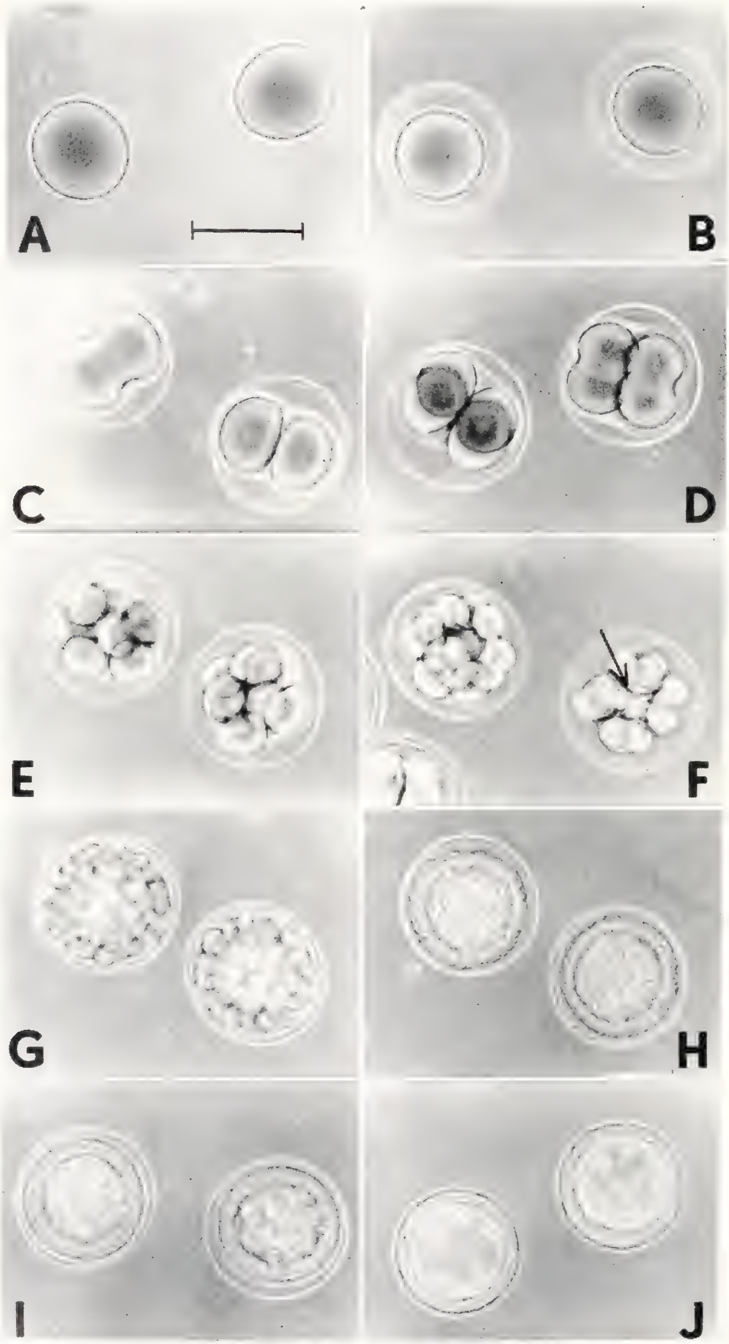
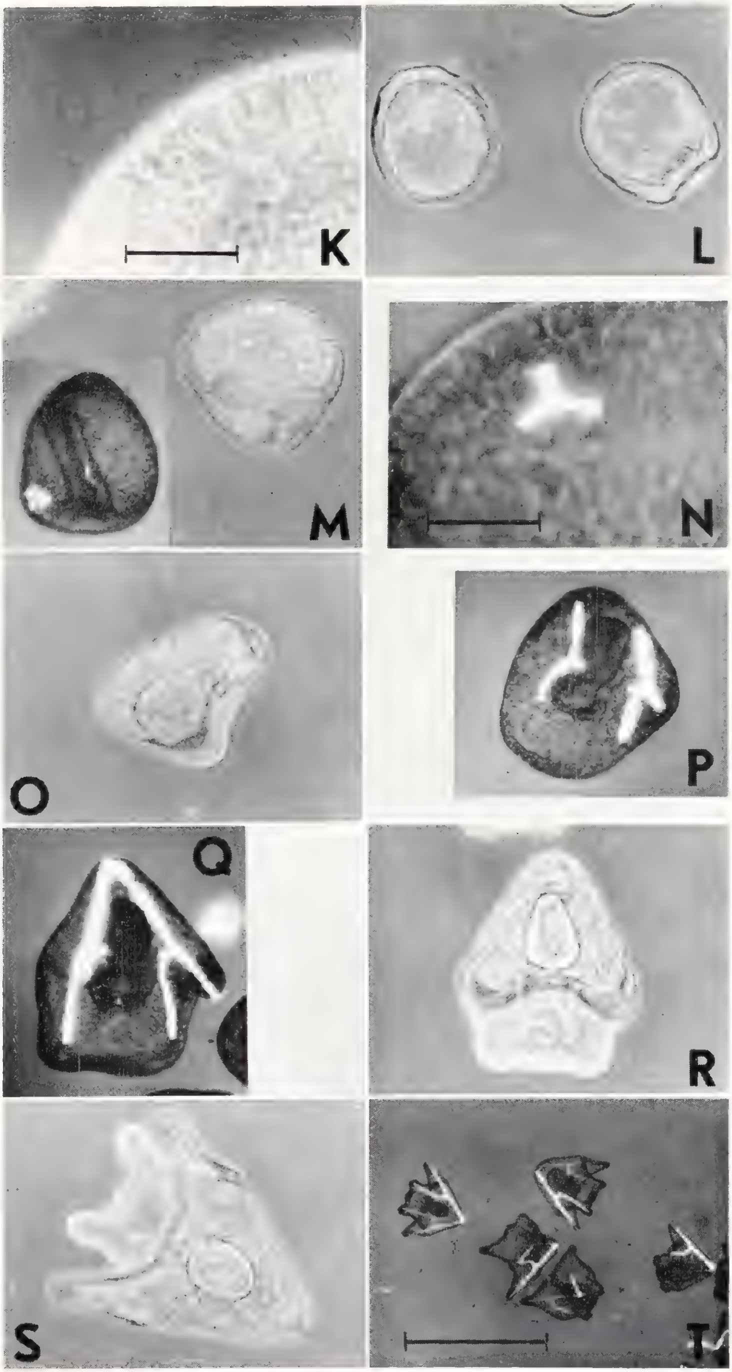


FIGURE 3. Developmental stages for fertilized eggs of *Strongylocentrotus droebachiensis* at 8° C; *A*—unfertilized egg (scale = 200 μ); *B*—5 minutes after fertilization; *C*—first division; 3 hr; *D*—second division, 5 hr; *E*—8-cell stage, $6\frac{1}{2}$ hr; *F*—16-cell stage, $8\frac{1}{2}$ hr (arrow: micromeres); *G*—early blastula, 15 hr; *H*—mid-blastula, 20 hr; *I*—cilia formation, beginning 24 hr; *J*—hatching, 30 hr; *K*—ciliated blastula, 32 hr (scale = 50 μ); *L*—early gastrula,



45 hr; *M*—late gastrula, spicule formation (inset: polarized light), 72 hr; *N*—tridentate spicule, polarized light, 72 hr (scale = 50 μ); *O*—prism stage, 96 hr; *P*—same as *O*, polarized light; *Q*—anal arm, polarized light, 5 days; *R*—same as *Q*, phase-contrast; *S*—oral arm, 7 days; *T*—pluteus, polarized light, 12 days (scale = 1 mm).

It is generally agreed that annual gonad development and spawning is frequently correlated with seasonal temperature maxima or minima. Boolootian (1966) remarks that the rate of temperature change may initiate gonad development while the reverse change may induce spawning, the gonad between these extremes requiring stronger stimuli for spawning the further it is from the time and temperature of natural spawning. If one accepts this general idea and applies it to *S. droebachiensis,* the lengthened breeding season of the Cape Cod Bay population may be reasonably rationalized. From Figure I it may be seen that in the fall the shallow waters of Cape Cod drop rapidly in temperature while the temperature change in the Gulf of Maine is moderated by its mass. This rapid drop-off in temperature, particularly in the Billingsgate Island area where most of the Cape Cod Bay animals were obtained, should influence early gonad development according to Boolootian's postulate. Both bodies of water reach temperature minima at the same time and warm in the spring at almost the same rates. Peak season and spawn-out thus should occur almost coincidently. Yearly seasonal variation would, of course, influence the termination of spawning. The differences in the two Maine populations reported by Harvey (1956) and Boolootian (1966) may simply reflect cooling and warming differentials. These arguments assume, of course, that an adequate and comparable food supply exists in all cases.

TABLE II

Temperature dependence of development (± 10%)

	0° C	4° C	8° C
2 Cell	8½ h.	5 h.	3 h.
4 Cell	13½ h.	8 h.	5 h.
8 Cell	17 h.	10½ h.	6½ h.
12 Cell	—	—	8¼ h.
16 Cell	23 h.	14 h.	8½ h.
32 Cell	30 h.	18 h.	11 h.
Cilia formation	65 h.	40 h.	24 h.
Hatching	80 h.	50 h.	30 h.
Invagination	5 d.	75 h.	45 h.
Mid-gastrula	7 d.	100 h.	60 h.
Spicule formation	8 d.	5 d.	72 h.
Prism stage	10 d.	6½ d.	96 h.
Anal arm	14 d.	8 d.	5 d.
Oral arm	18 d.	11 d.	7 d.
Maximum pluteus (without feeding)	1 m.	20 d.	12 d.

Since the Cape Cod Bay population does differ somewhat in habitat, breeding season, and egg appearance from its Maine counterpart, it might be mentioned at this point that both populations are unquestionably of the same species. Criteria outlined by Swan (1962) for distinguishing various *Strongylocentrotidae* indicate that the species clearly is *droebachiensis.* Out of roughly 5000 individuals examined over the past eight years, only one specimen, obtained in 1966 by commercial dredging off the coast of Maine, was tentatively identified as *S. pallidus.* The concept of physiological race (see for example Loosanoff and

Nomejko, 1951) also does not apply in this case since both populations spawn at essentially the same temperature, in spite of widely different summer temperature maxima, and animals from Maine subject to Woods Hole water temperatures ripen coincidently with the natural Cape Cod population.

Ecological considerations

The inability of *S. droebachiensis* to develop normally above about 10° C even under the most controlled laboratory conditions would indicate that this temperature represents an upper limit for larval development. The metamorphosed urchin and the adult clearly are exposed to temperatures 5–10° C warmer during mid-summer, but the distribution of *S. droebachiensis* correlates well with a 10° C upper limitation, for it is found in seas where the temperature during the early spring months rarely exceeds 6–8° C (Mortensen, 1943). South of Cape Cod the urchin is still found, but generally at moderate depths in the cold bottom waters. Since the larvae survive temperatures below zero quite well, the northern limitation in distribution would be determined only by actual freezing of coastal waters, permitting circumpolar distribution. But it is apparent from the laboratory behavior that the planktonic larvae of *S. droebachiensis* would not survive well in the more temperature surface waters to the south of Cape Cod.

Strongylocentrotus found in Cape Cod Bay occurs chiefly at Billingsgate Island, off Barnstable Harbor, and on the northeast jetty of the Cape Cod Canal. The latter two populations have become quite depleted recently, with no obvious cause. Past history of these beds indicates quite a variability in size-class and population density from year to year (John Valois, personal communication), something that would not be predicted for an indigenous steady-state population. One simple explanation for this variability is that a major proportion of settling larvae originate from the more northerly regions and are carried south to Cape Cod Bay; yearly variation in prevailing winds plus local environmental conditions would thus determine the success of an immigrant population. Such larval dispersal is now considered to be an important factor in genetic exchange in shallow-water marine populations (*cf.* Scheltema, 1971).

The extreme susceptibility of the newly-hatched blastulae to bacterial action, whether at the upper or lower reaches of its viable temperature range, would imply that survival of larvae in waters of high organic content would be substantially reduced. The sea water intake at the Marine Biological Laboratory is adjacent to a sewer outfall; as discussed above, larvae survive only when the water is rendered sterile or when sulfadiazine is added to the sea water. A similar situation might be envisaged in nature where organic pollution of a bay or estuary not subject to appreciable tidal action may result in mass mortality of these larvae. Of course, many other factors besides simple bacterial action may affect larval development; Wilson and Armstrong (1961) cite "biological differences" between various samples of sterile sea water, detected through their influence upon the embryogenesis of *Echinus esculentus.* Whatever the specific cause, the disappearance of large beds of *S. droebachiensis* in the vicinity of population centers should not be unexpected.

Timetable of development

The whole developmental program, at least through the pluteus stage, behaves as a tape played at various speeds depending upon temperature. Such proportionate time-temperature relationships are really not too surprising taken in the context of an average activation (Q_{10}) seen in other studies of echinoderm early development. Tyler (1936, 1942) observed no temperature optimum for either respiration or development, but rather a logarithmic time-temperature relationship apparently related to an over-all Q_{10} much as noted here for *S. droebachiensis.* Hoadley and Brill (1937) studied the timing of the first three cleavages of *Arbacia punctulata* and *Chaetopterus pergamentaceus* (annelid) and found an inverse logarithmic relationship with temperature.

What is somewhat surprising is the temperature range, specifically its lower limit. Perfectly normal pluteus larvae can be produced from eggs fertilized and grown entirely at *minus* 1° C. Considering modern observations on the role of microtubules in mitotic spindle structure (Inoué and Sato, 1967) or in later differentiation (Tilney and Gibbins, 1969) and the use by these and other workers of hypothermic treatment to abolish microtubules and thus stop mitosis or primary mesenchyme migration, it is interesting—to say the least—to have normal development at or below 0° C. The existence of diverse fauna at either low temperatures, high hydrostatic pressures, or both (*cf.* Sanders and Hessler, 1969), conditions under which microtubules or other low temperature and high pressure-sensitive organelles should be non-existent, may point to the evolution of organisms whose structural proteins associate more strongly or whose control mechanisms differ from the more commonly studied temperate littoral or terrestrial forms. In fact, mitotic spindle assembly at low temperate in *S. droebachiensis* confirms both stronger association and temperature modulation of assembly of spindle proteins (Stephens, 1972).

The use of S. droebachiensis *as an embryological material*

When obtained in shallow subtidal areas, *S. droebachiensis* has a breeding season quite comparable to its more commonly studied west coast relative, *S. purpuratus.* Simple precautions in laboratory handling make it as reliable as any other sea urchin. Its large, comparatively clear egg and its prolonged development are unique advantages; these facts, coupled with the large volume of obtainable eggs and good synchrony, would suggest that *S. droebachiensis* could be a valuable experimental organism.

Difficulty in laboratory use of *S. droebachiensis* as an experimental material can be easily attributed to several separate causes, all of them "violations" of the urchin's normal ecology. Upon collection, the animals must not be subjected to temperatures appreciably higher than those of their habitat; as with other sea urchins, warming will induce shedding of gametes. The same considerations must be made for the gametes themselves; shedding below 10° C is essential for high fertilization and synchronous development. During embryogenesis the temperature must remain constant and below 10° C for normal development and synchrony. After hatching the blastulae are extraordinarily sensitive to bacterial action and precautions must be taken to assure near-sterile conditions. As with most other embryos, overcrowding and lack of oxygen retard or arrest development; no more than 1 cm of sea water and cells one or two layers thick assure proper aeration through diffusion. Mechanical agitation or aeration of de-jellied eggs during the initial part of the first division (while the hyaline layer forms and the fertilization membrane hardens) generally results in cell clumping or even lysis. After the first cleavage, the cells are fairly insensitive to aeration or agitation in a reciprocating bath. All fertilization, washing, and transfer operations should be done with pre-chilled glassware to avoid temperature shock.

Choice of a pale yellow or near-colorless egg batch assures relative cell clarity. Slight compression (about 25%) is usually sufficient for observation of the mitotic apparatus, either in phase-contrast (by exclusion of granules) or in polarized light. A heat filter and temperature-controlled stage are essential if the cell is to be observed for any length of time. Reference to Figure 3 will show that most developmental events or relevant structural features are readily seen in *S. droebachiensis.*

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SUMMARY

1. Methods for obtaining viable gametes and embryos of the arctic-boreal sea urchin *Strongylocentrotus droebachiensis* are presented and practical suggestions are made regarding the suitability of this material for embryological use.

2. The useful breeding season extends from early January to mid-April for animals obtained from shallow subtidal regions of Cape Cod Bay or beginning roughly a month later for animals collected from the Gulf of Maine. The "season" can be extended by at least two months by holding the ripe animals at 4° C.

3. The time course for the first division and for development to the four-armed echinopluteus are given for various temperatures. Development time follows an inverse log relationship with temperature over the range of $-1°$ C to 9° C.

4. The susceptibility of the eggs and embryos to temperatures in excess of 10° C and of the hatched blastulae to bacterial action are discussed in regard to laboratory experimentation and the natural distribution of the organism.

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STUDIES ON THE DEVELOPMENT OF THE SEA URCHIN *STRONGYLOCENTROTUS DROEBACHIENSIS*. II. REGULATION OF MITOTIC SPINDLE EQUILIBRIUM BY ENVIRONMENTAL TEMPERATURE

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Modification of mitotic spindle birefringence through temperature change has proven to be a powerful tool for the investigation of spindle equilibrium and its thermodynamics. Such pioneering studies by Inoué and his collaborators (*cf.* Inoué, 1952a; Inoué and Sato, 1967) have revealed that spindle fibers are in a dynamic equilibrium with unpolymerized monomer and that shifts in this equilibrium can be correlated with spindle fiber growth and chromosome movement: a drop in temperature causes depolymerization of spindle fiber microtubules while warming causes reformation and growth. Analysis of this effect (Inoué, 1959 and 1964) indicates that the spindle monomer polymerizes with a high *positive* entropy change characteristic of hydrophobic bonding, that is, exclusion and *disordering* of many moles of bound water as one mole of monomer takes an *ordered* position in the polymer. Colchicine disrupts spindle structure, apparently through competitive binding with the free monomer (Inoué, 1952b) while D_2O enhances spindle fiber formation, presumably through some modification of water structures (Inoué, Sato, and Tucker, 1963). Thermodynamic analysis of the effects of D_2O indicates a significant change in the size of the pool of unpolymerized spindle monomer, in addition to changes in the thermodynamic parameters related to hydrogen *versus* deuterium bond energies (Carolan, Sato, and Inoué, 1966). This marked increase in pool size plus the apparent non-Newtonian mechanics needed for chromosome movement have served as focal points for criticism of the dynamic equilibrium theory (*e.g.*, Forer, 1969; McIntosh, Hepler, and Van Wie, 1969), but the mobilization of previously unavailable monomer to an active pool may have more than just secondary significance in the assembly and consequent disassembly of functioning spindle fibers and their mechanochemical role in chromosome movement.

Eggs of the sea urchin *Strongylocentrotus droebachiensis* can divide and develop to normal pluteus larvae at or below 0° C (Stephens, 1972), a temperature at which microtubules in most other organisms are non-existent. The existence, morphology, function, and control of the mitotic spindle at such low temperatures thus is of more than just academic interest. A comparison with mitosis at the higher end of the environmental temperature scale should shed some light into the dynamic nature of the spindle: is a spindle functioning at zero simply a diminished version of its warm temperature counterpart, or does the cell have some mechanism for temperature compensation?

MATERIALS AND METHODS

Experimental animals and gametes

The sea urchin *S. droebachiensis* was obtained from the Marine Biological Laboratory Supply Department and maintained at ambient sea temperatures in the running sea water system. The animals were fed *Laminaria*. Gametes were obtained and handled as described previously (Stephens, 1972), taking all possible precautions to maintain constant temperature during shedding of gametes, washing of eggs, fertilization, and development.

Mitotic apparatus isolation

Prior to fertilization, the heavy jelly coats of the eggs were removed by careful and rapid low pH treatment (Stephens, 1972). Spindles were isolated according to modifications of the basic hexylene glycol method of Kane (1962 and 1965). The eggs were fertilized and spun down in a hand centrifuge, the sperm suspension remaining in contact with the eggs for approximately one minute at 8° C or three minutes at 0° C. The eggs were then resuspended in 1 M glycerol containing 10 mM Tris. HCl, pH 8.0 (Kane, 1970) for 15 minutes at 8° C or for 45 minutes at 0° C, in order to prevent fertilization membrane and hyaline layer formation. The eggs were transferred *via* gentle hand-centrifugation to filtered sea water of appropriate temperature and incubated in a water bath. Immediately prior to metaphase (for metaphase time at various temperatures see Stephens, 1972) the cells were washed twice with isotonic NaCl-KCl in 19:1 ratio. After one wash with 1 M hexylene glycol buffered at pH 6.4 with 10 M phosphate, the cells were lysed in fresh hexylene glycol medium by vortex-mixing. In all of the above washes, the reagent volumes were ten times the egg volume; one ml of eggs was typically used. The temperature of the NaCl-KCl and hexylene glycol medium was determined by the experimental design. Immediately upon release of the mitotic apparatuses, the solution was chilled in ice. The spindles were then sedimented at 2000 × *g* for 2 minutes in a clinical centrifuge and washed three times *via* centrifugation with 5 ml of ice-cold isolation medium. The spindles were resuspended in about 0.1 ml of isolation medium for observation.

Microscopy

Observations in polarized light were made within a Leitz Ortholux Pol microscope equipped with a Xenon light source, Baird-Atomic 546 nm interference filter, heat filter, and selected strain-free optics. A Brace-Koehler compensator of 18 nm retardation was used for measurement of birefringence. Photographs were taken with Kodak Panatomic-X film developed in Diafine developer. Phase-contrast observations were made with Zeiss optics and photographed on Panatomic-X film developed in Kodak Microdol-X. When both phase-contrast and polarization micrographs were necessary, a phase annulus was placed in the position normally occupied by the lamp iris in the base of the Ortholux microscope and a pre-centered phase-contrast objective was swung into place by means of a pre-centered turret. It was thus possible to switch from polarized light to phase-contrast and back without moving the specimen or removing the strain-free polarizing condenser. The analyzer, of course, was removed for phase-contrast observation, but (given a sufficiently strain-free phase objective) the analyzer could be used to obtain a combined polarization-phase-contrast image, useful in visually relating mass and birefringence.

Cells at the appropriate stage were placed on a water-cooled temperature controlled slide, the temperature of the specimen being taken as the average of the influx and efflux temperatures. Coverslip spacers, 0.18 mm thick, were used to prevent compression; when compression was desired, no spacer was used and the fluid was drawn from beneath the single coverslip with filter paper until the desired degree of compression was obtained.

Gel electrophoresis

Mitotic apparatus preparations were analyzed electrophoretically using 5% polyacrylamide gels containing sodium dodecyl sulfate (SDS) by methods devised by Shapiro, Vinuela, and Maizel (1966) and modified by Weber and Osborn (1970). The gels were stained with Fast Green (Gorovsky, Carlson, and Rosenbaum, 1970) by appropriately replacing the Coomassie Blue dye in the Weber-Osborn formulation, and then quantitated by densitometry. The position of tubulin in the mitotic apparatus preparations was established by a parallel gel containing the pure protein obtained from sperm flagellar outer fibers (Stephens, 1970).

Tubulin synthesis

Tubulin synthesis at 0° C and at 8° C was monitored at ten successive intervals during the first division by pulse-labeling 0.2 ml of eggs with 5 μc of ^{14}C-leucine in 2.0 ml of sea water, separating the tubulin from 1 mg of total cell homogenate with SDS-polyacrylamide gel electrophoresis, and then either eluting the protein from the gel and counting by scintillation methods or by direct autoradiography of the longitudinally-sliced gel (Fairbanks, Levinthal, and Reeder, 1965). Full details of these methods are outlined in another paper of this series (Stephens, in preparation).

Chemicals

Uniformly-labeled ^{14}C-leucine, with a specific activity of 312 mC/mmole, was obtained from Schwarz/Mann, Orangeburg, New Jersey. Colchicine was obtained from City Chemical Corporation, New York, New York.

RESULTS

Observation and isolation of the first division mitotic spindle at 8° C and 0° C or after temperature jumps to 20° C

Two types of determinations were made. Cells were fertilized and grown at either 0° C or at 8° C until metaphase; the spindle birefringence was measured, the cells were brought to 20° C, and the birefringence was remeasured. As an alternative to these *in vivo* determinations, the mitotic apparatus was isolated at either 0° C, 8° C, or at 20° C after prior growth to metaphase at either of the former temperatures; the birefringence of the spindle isolate was then determined *in vitro*. No significant birefringence differences were noted between the *in vivo* and the *in vitro* measurements.

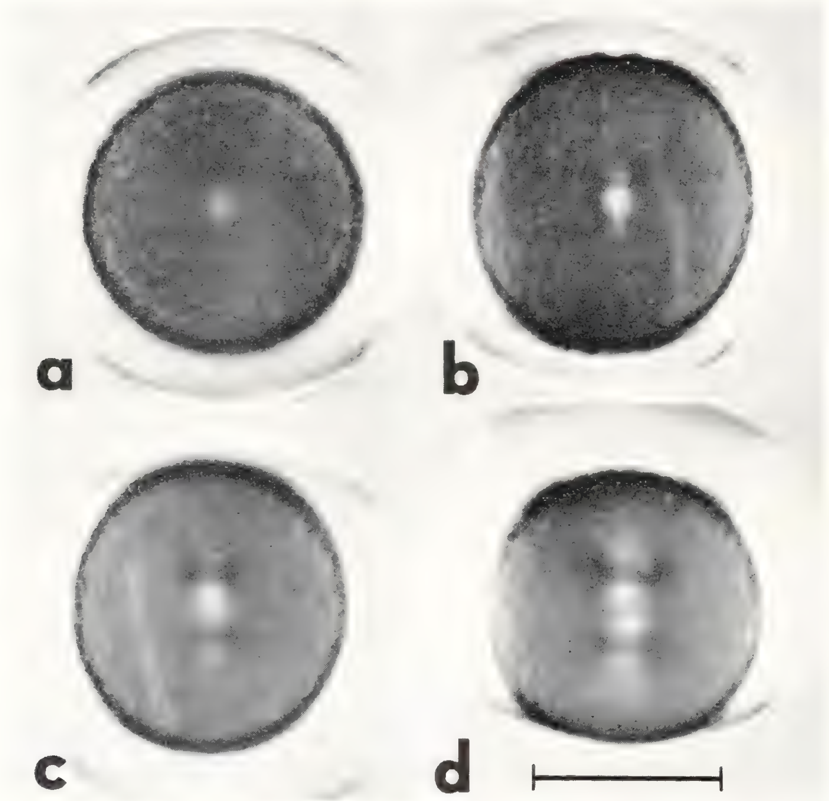

FIGURE 1. Eggs at metaphase grown at two specific environmental temperatures and transferred to 20° C, (a)—cell fertilized and grown at 0° C, retardation = 1 nm; (b)—same as (a) but brought to 20° C at metaphase, retardation = 2.5 nm; (c)—cell fertilized and grown at 8° C, retardation = 2.7 nm; (d)—same as (c) but brought to 20° C at metaphase, retardation = 4.5 nm, scale = 100 μ.

At 8° C the mitotic spindle *in vivo* was of the normal amphiastral type, measuring 30 μ from pole to pole, and having a metaphase retardation of 2.75 ± 0.25 nm, measured in the central spindle (Fig. 1c). When these cells were brought to 20° C, the birefringence increased markedly, reaching a maximum retardation of 4.50 ± 0.25 nm within two minutes. Large asters formed and the spindle "enlarged" somewhat, measuring 40–50 μ from pole to pole (Fig. 1d). Transfer of cells from 8° C to 0° C caused no obvious morphological changes, but reduced the retardation of the central spindle region to 1.5 ± 0.2 nm. Asters were still present but very weakly birefringent. When spindles were isolated at 8° C and at 20° C after growth to metaphase at 8° C, essentially identical results were obtained but the differences in both morphology and birefringence were substantially more obvious (Fig. 2b and 2d compared with Fig. 1c and 1d).

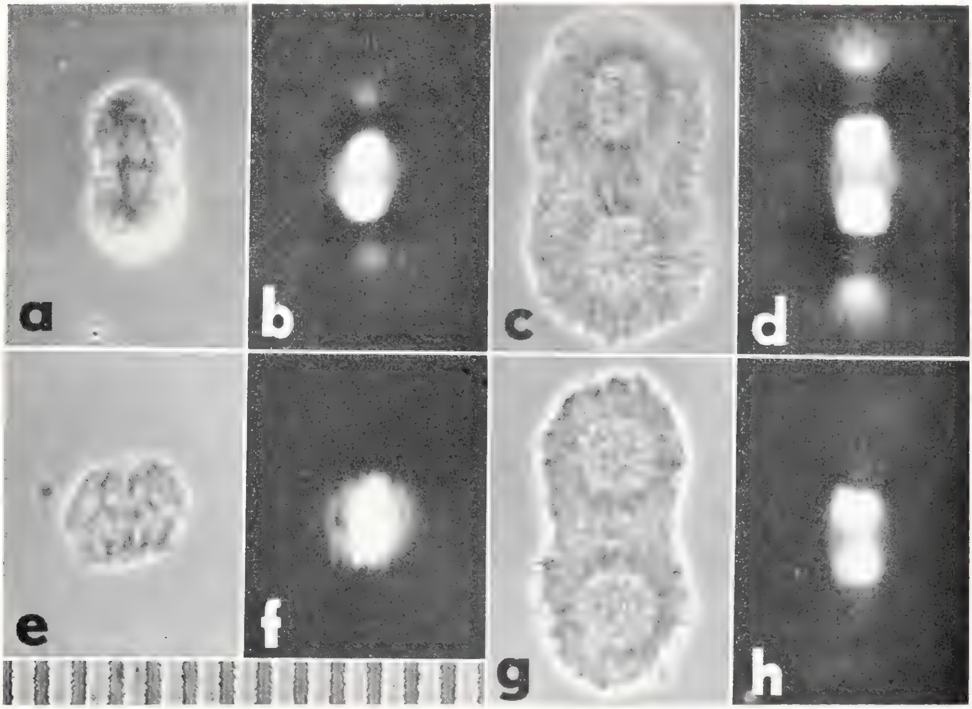

FIGURE 2. Spindles isolated at metaphase, grown at two specific environmental temperatures and transferred to 20° C, (a)—cell fertilized and grown at 8° C, spindle isolated at metaphase, phase-contrast optics; (b)—same as (a), polarization optics; (c)—cell fertilized and grown at 8° C, but spindle isolated after transfer to 20° C, phase-contrast optics; (d)—same as (c), polarization optics; (e)—cell fertilized and grown at 0° C, spindle isolated at metaphase, phase-contrast optics; (f)—same as (e), polarization optics; (g) cell fertilized and grown at 0° C, but spindle isolated after transfer to 20° C, phase-contrast optics; (h)—same as (g), polarization optics; Scale intervals = 10 μ.

At 0° C no "spindle" as such was observed. Rather, an apparent *uniaxial sphere* with a diameter of about 30 μ and a retardation of 0.95 ± 0.2 nm was typically observed; no distinct poles or asters were evident (Fig. 1a). Transfer of these cells to 20° C resulted in the enhancement of central spindle birefringence to a value of 2.3 ± 0.25 nm, a pole to pole distance of 40–50 μ, but only barely perceptible growth of weakly birefringent asters (Fig. 1b). Cells grown at 0° C and brought to 8° C had a central spindle retardation of 1.5 ± 0.2 nm. Isolation of the 0° C spindle revealed that the uniaxial sphere seen *in vivo* was a totally anastral spindle (Fig. 2e and 2f) while the spindle of cells grown at 0° C and brought to 20° C consisted primarily of birefringent chromosomal and continuous spindle fibers with an astral region of considerable mass but nearly immeasurable birefringence (Fig. 2g and 2h).

Execution of the "growth phase" of the above experiment at intermediate temperatures resulted in the production of intermediate spindles with respect to both birefringence and morphology not only at the temperature of growth but also after the temperature jump.

These data are consistent with the hypothesis that there is more spindle fiber monomer available in cells grown at 8° C than 0° C since the birefringence of a spindle grown at 8° C and transferred to 20° C is twice that for a spindle grown at 0° C and similarly transferred or, alternatively, the birefringence of a spindle grown at 8° C and brought to 0° C is at least 50% higher than that of a spindle grown and observed at 0° C. This argument makes use of only the central spindle birefringence; the amphiastral nature of the 8° C spindles provides even more evidence for this conclusion. Table 1 summarizes the above birefringence data and includes an estimate of the relative amount of tubulin and 22S protein obtained from the spindles of an equal number of eggs grown at 0° C and 8° C, or grown at these temperatures and transferred to 20° C for isolation. Figure 3 illustrates a typical SDS-acrylamide gel electrophoretic pattern obtained from *S. droebachiensis* spindle isolates, accompanied by parallel gels of a whole egg homogenate and of sperm flagella axonemes. Only the tubulin fraction was found to be consistently proportional to the birefringence of the spindle. Admittedly, this correlation of birefringence with tubulin content represents only a first approximation since no correction has been applied here for geometrical differences in the central spindle regions of the various spindle preparations. The other components, however, particularly the 22S and 2.5S proteins (Stephens, 1967), varied directly with the spindle volume and inversely with the degree of washing. The stoichiometry of mitotic apparatus components and their possible role in mitosis

TABLE 1

Birefringence and tubulin content data for spindles at various growth and isolation temperatures

Temperature		Birefringence (nm retardation*)	Relative tubulin†	Relative 22S†
Growth	Isolation			
0°	0°	0.95	0.3	0.6
0°	8°	1.50	—	
0°	20°	2.30	0.9	3.1
8°	0°	1.50	—	
8°	8°	2.75	1.0	1.0
8°	20°	4.50	1.9	4.2

* Measured in the central spindle: Retardation = Compensator Retardation × sin 2θ, where θ = difference between maximum background compensation and maximum spindle compensation angles, with specimen at 45°. Error is ± 5%.

† Relative to that obtained from 8° C spindles isolated at 8° C.; determined microdensitometrically from the tubulin or 22S bands obtained from the SDS-acrylamide gel electrophoresis of spindles from equal numbers of eggs.

are currently being investigated further, but the results of this present work indicate that the majority of them arise by a "sponge" effect due to trapping of cytoplasmic components. This is quite consistent with the recent finding that the 22S and 2.5S protein "mitotic" components are constituents of the yolk granules or other particulate fractions and not of the spindle fibers themselves (Burns and Kane, 1971).

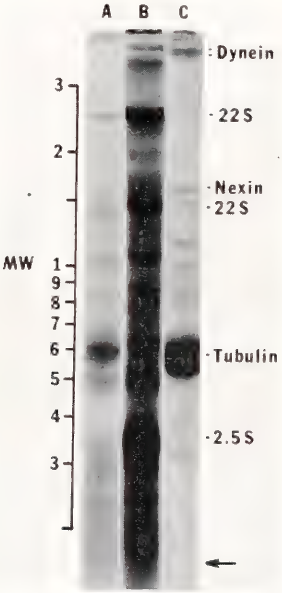

FIGURE 3. Comparative SDS-polyacrylamide gels of mitotic apparatuses isolated at 8° C, (A), whole egg homogenate (B), and sperm flagellar axonemes (C); flagellar components from Stephens (1971); 22S and 2.5S components from calibration with yolk particulate fraction (*cf.* Burns and Kane, 1971); lower molecular weight (MW) cycle = 20,000 to 100,000; upper cycle = 100,000 to 400,000 (not accurate above 250,000 and below 30,000 due to deviations characteristic of 5% gel systems). No dynein is observed in the mitotic apparatus isolates, although a *single* dynein band is apparent in the whole egg homogenate, corresponding in mobility to the higher molecular weight dynein subunit in flagella. Arrow indicates the position of the original marker-dye front.

Electrophoretic separation and subsequent counting of tubulin from homogenates of eggs pulse-labeled throughout the first division showed no significant difference in the amount of total tubulin synthesized at any given point in the cell cycle at either 0° C or at 8° C; the overall pattern of protein synthesis was also identical in both cases.

Second division metaphase at a new environmental temperature

In the next division of the cell, is the amount of spindle monomer available for use influenced by temperature conditions during the first division?

When cells were grown at 0° C through cytokinesis and then transferred to 8° C, mitosis proceeded precisely according to an 8° C timetable for the second division (*cf.* Stephens, 1972). Similarly, cells grown at 8° C and transferred to 0° C just following cytokinesis behaved as typical 0° C cells in second division. Observation of the second division spindle under these respective conditions showed no significant deviations in spindle fiber birefringence or overall morphology when compared to spindles from eggs grown at a single temperature. Thus, temperature conditions of the first division have no influence on spindle birefringence and morphology, or on the time course of the second division. Further division results in somewhat smaller spindles and reduced asters, making any later comparisons difficult.

The influence of temperature changes during the first division upon the character of the metaphase spindle

When does the cell "mobilize" the prescribed amount of spindle monomer for its charcteristic spindle?

Eggs from the same female were fertilized and grown at either 0° C or 8° C and then transferred to the opposite temperature at various points in the premetaphase cell cycle. The type of spindle that developed was judged by both size and birefringence. In the following description, a "0° C spindle" will refer to a uniaxial sphere with a retardation of about 1 nm while an "8° C spindle" will refer to an amphiastral spindle with approximately 3 nm retardation (for example, Fig. 2B *versus* Fig. 2F).

At any point up to and including nuclear membrane breakdown, cells fertilized and initially grown at 8° C and then transferred to 0° C developed typical 0° C spindles; transfer during early prophase brought about intermediate types of spindles having a retardation of about 2 nm and developing small asters. Few of these cells underwent cytokinesis according to the 0° C timetable when the transfer took place at or after nuclear membrane breakdown; rather, they underwent a highly asynchronous, multipolar second division.

Cells fertilized and grown at 0° C and then transferred to 8° C at any point up to and including nuclear membrane breakdown produced spindles characteristic of cells grown entirely at 8° C. The remainder of the division cycle proceeded according to an 8° C time scale and normal cytokinesis took place. Transfer during early prophase, as in the reverse transfer cited above, resulted in the formation of intermediate spindle types.

When cells are properly flattened, nuclear membrane breakdown and early prophase can be observed with great precision through optical sectioning. From the foregoing observations, the "switch point" in determining the type of spindle that will form appears to be early in prophase, when the asters are actually in the process of forming. Differences in synchrony and temperature variations during observation make it impossible to be more exact.

DISCUSSION

Implications for mitotic equilibrium

A number of new facts can be immediately pointed out as being revelant to mitosis in its broadest sense:

1. *Spindle fibers can and do exist at 0° C* as evidenced by a typical 0° C spindle (Fig. 2e and 2f) or by the fact that an 8° C spindle brought to 0° C loses less than half of its birefringence (Table I).

2. *Functional spindles of the same division cycle in the same organism can be either amphiastral or anastral* (Fig. 2a *versus* 2e); temperature-induced growth of the anastral type results principally in the increase of chromosomal and continuous fiber birefringence (Fig. 2h).

3. Substantially more spindle monomer is available at 8° C than at 0° C, whether judged by spindle birefringence or tubulin content (Table I), demonstrating that *monomer pool size can be naturally varied.*

4. *The monomer is apparently made available at one discreet point in the cell cycle,* with the amount being determined by the temperature during *early prophase.*

5. *No temperature compensation appears to exist;* rather, the process of activation or mobilization during early prophase behaves like a biological process whose temperature coefficient differs markedly from the average of the cell as a whole.

The ability of *S. droebachiensis* to form spindle fibers at such extremely low temperatures very likely indicates the evolution of a tubulin whose subtleties in primary structure permit a substantially stronger degree of association than that from animals living at higher temperatures. In this regard it might be pointed out that isolated spindles of *S. droebachiensis* retain their birefringence for significantly longer periods of time when stored at room temperature in isolation medium than do those from *Arbacia punctulata,* a sea urchin having a 15° C higher temperature range. Kane and Forer (1965) report a retardation of 3–4 nm for spindles isolated at room temperature from the latter urchin; approximately half of the birefringence is lost from such spindles in one hour at room temperature. Spindles from *S. droebachiensis,* however, have this same retardation when isolated at 8° C and they lose birefringence at about 25% per hour at room temperature. The response to colchicine is likewise somewhat slower; concentrations roughly twice those used by Inoué (1952b) were needed to decrease birefringence in *S. droebachiensis* at a rate comparable to those which he achieved for *Chaetopterus.* Confirmation of the hypothesis of a more strongly-associating mitotic tubulin must await *in vitro* comparison of *S. droebachiensis* tubulin with that from another species whose spindle fibers are substantially more cold labile.

The functional existence of an anastral spindle in the form of a uniaxial sphere at 0° C would indicate that the astral regions of the more typical sea urchin spindle would have little function in mitotic movement *per se.* Inoué (1964) has observed that birefringence is strongest near poles and centromeres, the "orienting centers" of his dynamic equilibrium theory. Brief reference to Figure 2 illustrates that the birefringence is strongest between the chromosomes and the "centrosphere," with the "0° C to 20° C" spindle (the condition with apparently the least amount of polymerizable spindle fiber material) devoting essentially all of its oriented material to the formation of chromosomal and continuous spindle fibers (Fig. 2h). With presumably a great deal more available spindle precursor, the "8° C to 20° C" spindle apparently can afford to form large asters (Fig. 2d). If spindle material is conserved during development, and not appreciably synthesized, spindles should get smaller with each successive division; Harris (1962) not only notes that this does happen but she also observes that spindles become anastral. Harris speculates that perhaps the possession of asters during the first several divisions may be a specialization used by large cells to guarantee equal cytokinesis but the fact that the totally anastral 0° C first division spindle in *S. droebachiensis* (an egg 160 μ in diameter) partitions the egg quite equally would argue against this point. A striking feature of the temperature-induced "giant" spindles of *S. droebachiensis* is the huge "centrosphere," strikingly visible in the phase-contrast micrographs (Fig. 2c and 2g); polarized light images indicate no clear-cut single "center" as is so apparent in the untreated spindles at 8° C (compare Fig. 2d with 2b). Ultrastructural studies of high and low-temperature spindles, with particular emphasis on the centrosphere region are currently underway (Stephens, in preparation).

The study by Carolan, Sato, and Inoué (1966) of the effects of D_2O on metaphase-arrested *Pectinaria gouldii* oocytes indicated that this agent reversibly increases the amount of spindle precursor available for polymerization, in addition to modifying the thermodynamic parameters of association. Though not so stated, the mobilization of polymerizable spindle fiber material prior to metaphase and the reverse process thereafter could provide the motile force needed by the dynamic equilibrium theory. Indeed, this change in "pool" is necessary for the theory since it simultaneously assumes that the equilibrium constant is truly constant and that at any given moment there is an upper limit to the amount of available spindle fiber precursor. Otherwise, how can the equilibrium be dynamic? Arguments can be made that D_2O effects a multitude of things in the cell, that arrested metaphase is a unique situation, and that response to any agent is dependent upon the stage of mitosis, but the evidence presented here for environmentally-controlled or modified spindles is not subject to these arguments. The overall pool size is clearly different in the two temperature cases.

The fact that some temperature-dependent process during prophase (by definition, the time during which the spindle is assembled) determined how much total spindle material is mobilized would point to the possibility that there exists, in the cell, sites possessing this same potential which may be active in either a forward or reverse direction during mitotic movement. Thus some simple biochemical process may locally activate or deactivate spindle material during the mitotic cycle, bringing about the localized polymerization-depolymerization postulated in the dynamic equilibrium theory (Inoué, 1964). It may be significant that during mid-to-late anaphase, in spite of the presence of "orienting centers" at the poles and kinetochores, increase in temperature causes little change in birefringence in either 0° or 8° C cells, impling an inactivation of previously-available spindle fiber monomer.

Over an organism's normal temperature range, the developmental processes generally follow a rate *versus* temperature relationship characteristic of the sum total of enzyme rates in the cell. Early studies of cleavage rates (Hoadley and Brill, 1937), first division events (Fry, 1936), or respiration during embryogenesis (Tyler, 1942) at various temperatures within the viable range indicated that the log of the respective rates varied inversely with temperature. The whole pattern of early development in *S. droebachiensis* follows the same proportionality sequence regardless of temperature; only the relative rates vary (Stephens, 1972).

It is in this one unique case—spindle formation—where a developmental event apparently has a substantially different activation energy from the "average" process in the cell and where the product of this developmental event, the spindle fiber material, itself undergoes a temperature-dependent polymerization process. Thus, even though the overall pattern of protein synthesis and the proportionality of the timetable of mitotic events is independent of temperature, this one event can be "dissected" from the rest.

Some thoughts on the mechanism of mitosis

The purpose of this paper is to present data, not dogma. But since the interpretation of this data must eventually be evaluated in the context of existing theories of mitosis, comment on its consistency with such concepts seems in order.

It has already been pointed out that the demonstration of a natural change in pool size and the consequent implication of some prophase event mobilizing polymerizable spindle fiber material are findings which support and strengthen the dynamic equilibrium theory of Inoué. That a similar mobilization or demobilization may take place locally during anaphase movement is admittedly speculation, but it is an idea that is central to the Inoué mechanism. In addition, the results presented here demonstrate that the amount of tubulin obtained from a spindle is directly proportional to the birefringence, while other components vary with spindle size or degree of washing. This observation should lay to rest the criticism that birefringence is not necessarily a measure of oriented microtubule material (*cf.* discussion in Forer, 1969). The present experiments do not contribute any further insight into the mechanism of force production by polymerization and depolymerization of microtubules, a seeming violation of Newtonian physics. In this regard, however, Inoué, (1952b) clearly demonstrated that, in *Chaetopterus*, when one pole is anchored to the cell cortex, application of colchicine in low concentration brought about simultaneous diminution of birefringence and chromosome movement toward the cell cortex, indicating the depolymerization can result in a "pulling" force. Induced growth of spindles, whether metaphase-arrested or not, either by heat or D_2O, results in a marked increase in interpolar distance (Inoué and Sato, 1967; this report, Fig. 2a *versus* 2c), so there is little doubt that increased polymerization can result in a "pushing" force. It is still not clear, however, exactly how this force can be generated. Perhaps some cyclic anchoring mechanism involving "matrix" material around the tubules is involved or perhaps local conformational changes within the tubule substructure (*e.g.*, Thomas, 1970) may allow addition or removal of spindle monomers in the center of a spindle fiber without affecting its integrity, a notion which at the time that it was proposed (Inoué, 1964) was thought to be implausible by many.

Various sliding filament mechanisms have been proposed for chromosome movement (*e.g.*, Bělař, 1929; Bajer, 1968; Subirana, 1968; McIntosh, Hepler, and Van Wie, 1969). The mechanism of McIntosh *et al.* (1969) is a variation on a theme by Huxley (1969) involving polarized chromosomal and continuous spindle fibers with appropriately oriented cross-bridges; the others are less specific and hence far less testable. Mechanically and mechanistically, sliding filaments offer an attractive hypothesis, but numerous questions must still be answered.

In order to slide over one another, filaments must be close enough to do so and must be attached to the proper places; spatial arrangement and microtubule continuity are major unresolved problems at this point (see through discussion in Nicklas, 1971). Even where appropriately connected and arranged microtubules are close enough to interact, they must have something to interact with; both Wilson (1969) and McIntosh, Hepler, and Cleland (1971) present evidence for specific bridges, but more conclusive evidence is needed on this point. Where interaction is postulated to be with adjacent cytoplasmic material (Subirana, 1969), where is and what is this material? What prevents sliding of filaments before metaphase and what controls their simultaneous movement and disappearance during anaphase? Poleward particle movement (Bajer, 1958) or poleward movement of a UV-irradiated spot (Forer, 1965), both prior to metaphase and both with rates comparable to anaphase movement, indicate that a great deal might be taking place before the actual movement of chromosomes, a time when sliding filaments should be inactive. Under any sliding mechanism, how do spindles that *eliminate* chromosomes function (Metz, 1938)? Perhaps the chromosome should not be treated simply as a passenger!

Regarding the temperature-equilibrium observations made here, one might ask the question of how a spindle with sliding filaments can still function quite normally with half of its usual filament material? Intuitively, one might expect cells to have evolved a true temperature compensation mechanism wherein *more* spindle material is mobilized at lower temperatures to offset the depolymerizing effect of such temperatures. It is also not easy to visualize how mitotic movement *via* an active sliding process would be consistent with the observations of chromosome movement by simple depolymerization (Inoué, 1952b) or the change in interpolar distance by heat or D_2O treatment (Inoué, and Sato, 1967) discussed above.

Probably the only indisputable conclusion that can be drawn from the foregoing discussion is that a great deal more work on this problem is warranted.

On the possible nature of the mobilization process

It is generally agreed that the major components of the mitotic apparatus (in eggs at least) pre-exist in the cell, to be utilized at prophase (Mazia, 1961), and hence the idea of a mobilization of spindle fiber material is clearly not new. Its final proof must still await a demonstration of differences between polymerized spindle material and that from the postulated inactive pool. Several plausible biochemical mechanisms come immediately to mind. Guanosine triphosphate is a necessary cofactor for polymerization of flagellar tubulin (Stephens, 1968 and 1971); possibly the addition of the nucleotide to the tubulin or the phosphorylation of bound guanosine diphosphate is the activation step. Cyclic changes in -SH content of both the whole cell and the mitotic apparatus are well documented (Mazia, 1961) and experimental modification of -SH balance can cause reversible depolymerization of the spindle (Stephens, Inoué, and Clark, 1966). Either the formation or the breakage of intramolecular disulfide bonds may cause sufficient conformational change to promote markedly different association properties for the oxidized or reduced form. Amination, glucosylation, phosphorylation or other post-synthesis modification of amino acid side chains would offer another possible activating step. Peptide cleavage, as in the fibrinogen-fibrin transformation, is an unlikely possibility since such a process during mitosis would very likely need to be reversible. The possibility exists that other proteins besides tubulin "copolymerize" in the microtubule; the presence or absence of these postulated cofactors might in turn determine microtubule polymerization initiation and kinetics. Exactly what this cellular control process involves thus poses a crucial problem; its nature is fundamental to the whole question of microtubule polymerization and function, and perhaps to mitosis itself.

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SUMMARY

1. The assembly and function of the mitotic apparatus in first division eggs of the sea urchin *Strongylocentrotus droebachiensis* were studied at 0° C and at 8° C by polarization microscopy *in vivo* and after isolation in hexylene glycol at controlled pH.

2. No differences in the amounts of total tubulin synthesized over comparable periods of the cell cycle were observed.

3. Mitotic apparatuses from a cell grown at 0° C are anastral, while those grown at 8° C are amphiastral.

4. At a 0° C growth temperature only about one-half of the spindle fiber monomer is available for polymerization as at 8° C, indicating a natural variation in pool size.

5. The amount of spindle monomer made available to the usable pool is specified only by the temperature during early prophase, with temperature prehistory having no effect.

6. The temperature coefficient for this apparent activation differs markedly from that of the mitotic process as a whole.

7. The amount of tubulin obtained from an isolated mitotic apparatus, as determined by electrophoretic separation of tubulin on SDS-acrylamide gels, is directly proportional to its measured retardation, implying that birefringence is a true measure of oriented microtubules.

8. These results are compatible with the mitotic dynamic equilibrium theory of Inoué and complement this theory by providing evidence for natural variation in pool size (and thus for the potential local mobilization of monomer during anaphase movement) and by providing a direct correlation between birefringence and tubulin content of spindles.

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ACCUMULATION OF FREE FATTY ACIDS FROM SEA WATER BY MARINE INVERTEBRATES

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Numerous investigators have reported uptake of dissolved free amino acids, glucose, and other small organic molecules from sea water by marine invertebrates (reviewed in Stephens, 1971). Other than the brief observations by Southward and Southward (1970) on uptake of palmitic and butyric acid by three species of Pogonophora, there are no published investigations on the ability of free-living marine invertebrates to take up fatty acids from sea water.

Dissolved fatty acids are available in the marine environment. Slowey, Jeffrey and Hood (1962) found fatty acids of 12–18 carbon number in ethyl acetate extracts of millipore filtered sea water taken from the Gulf of Mexico. Free fatty acids, long chain hydrocarbons, fatty acid esters, sterols, and many other substances, including phosphorus and nitrogen containing compounds which may have been phospholipids, were identified by Jeffrey, Pasby, Stevenson and Hood (1963). Total concentration of ethyl acetate/petroleum ether-extractable substances in these inshore Gulf samples was 4–14 mg/liter. Jeffrey (1966) hydrolyzed lipids and identified acids having less than 10 to 22 carbon atoms, with up to 6 unsaturated linkages in the longer acids. Total dissolved lipid concentrations ranged from 0.5 to 6.0 mg/l, the lower values being in the offshore Gulf samples. Williams (1965) found only 1–9 μg/l of dissolved fatty acids in samples from within and just outside of Puget Sound.

In view of the existence of fatty acids in the marine environment, it seemed desirable to determine if soft-bodied marine invertebrates other than pogonophorans could take up and assimilate free fatty acids. When preliminary experiments demonstrated the ability of several polychaetous annelids to accumulate C^{14} in the form of palmitic and oleic acid, it became of particular interest to determine whether a "saturable" uptake system was involved, in which the velocity is limited by the availability of a finite number of transport sites. Inhibitory interactions among fatty acids were investigated to cast light on the specificity of the uptake mechanism. Labelled animals were extracted and fractioned into the major biochemical classes by solvent partition and thin-layer chromatography in order to determine if the free fatty acids participate in energy metabolism and synthetic pathways.

Accumulation of a radioactive label is not adequate evidence that net accumulation of the compound occurs. When exchange diffusion or label exchange are operating, an organism may show initial accumulation of label even though the compound is leaking out faster than it is being taken up. The disappearance of unlabelled free fatty acid from the medium was monitored in order to check for this possibility.

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Since uptake rate is a function of substrate concentration, once this function has been defined, data on the concentration of fatty acids in the natural environment of the organism can be used to make an estimate of the significance of the uptake process relative to the metabolic requirements of the organism. In view of the variability of fatty acid levels reported in the literature and the failure of these reports to specify how much of the fatty acid was in the free or combined form, it was deemed desirable to collect sea water samples from the habitat of the organism being studied and assay them for free fatty acids.

MATERIALS AND METHODS

Stauronereis rudolphi (Della Chiaje) (Annelida, Polychaeta, Dorvilleidae) is especially plentiful during the summer and fall months among the mats of green algae and associated detritus that coat pilings and logs floating in Los Angeles Harbor. The animals burrow into the substrate and ingest it, but do not maintain permanent burrows (Reish, 1959). Specimens collected from the harbor were kept at 20° C in gallon jars of aerated and continuously filtered sea water and fed pellets of dried alfalfa ad lib. Worms were used within six weeks of collection.

Nainereis dendritica (Annelida, Polychaeta, Orbiniidae) abounds at and below the low tide line on exposed sandy beaches. Organisms were collected from under roots of *Phyllospadix* one mile south of Corona Del Mar and kept at 15° C in a large bucket of aerated sea water with several inches of sand in the bottom. Specimens were used within three weeks of collection.

Oleic, palmitic, stearic and linoleic acids labelled in the 1-carbon position with C^{14} were supplied by Amersham/Searle together with data attesting to at least 98% radiochemical purity. The claimed 99% purity of uniformly labelled palmitic acid from the same company was affirmed by thin-layer chromatography (TLC) on silica gel in pertoleum ether, ethyl ether, acetic acid: 70:30:2 (v/v/v). Caproic acid-1-C^{14} was obtained from Calbiochem and the acetic acid-1,2-C^{14} was supplied by Volk Radiochemical.

Accumulation of C^{14}-labelled free fatty acids

Glass experimental vessels were coated with "Siliclad" (Clay-Adams, Inc.) to minimize fatty acids sticking to the glass. Teflon beakers were also used in some experiments.

Artificial sea water (M. B. L. S. W.) made up in distilled water from reagent grade salts according to the standard Marine Biological Laboratory, Woods Hole, formula (Cavanaugh, 1964) was millipore filtered twice just before use. Animals were allowed to remain at least 24 hours in clean M. B. L. S. W. to empty the gut before transfer to the experimental beakers. Small worms such as *Stauronereis* were incubated in groups of 1 to 2 dozen so that each beaker contained about 300 mg of animal material. Enough radioactive fatty acid was introduced into each 50 ml beaker of M. B. L. S. W. to give an activity of 20 μc/l. Carrier for the radioactive stock was 0.1 ml of 85% ethanol. Unlabelled fatty acid (supplied by Calbiochem) was added to bring the solution up to the desired concentration.

After incubation, rinsing and weighing, the worms were dropped into scintillation vials containing 10 ml of scintillation fluid (6 grams PPO/l toluene, 2 parts added to 1 part Triton-X detergent) and left 2 days before counting. If quenching was too great, the extract was diluted 10 × before counting. This method extracted 97% of the radioactivity in the worms. Larger invertebrates were digested with formic acid. The vials were counted in a Beckman CPM-100 liquid scintillation spectrometer. Efficiency of C^{14} counting of experimental samples was approximately 80%.

Assimilation of palmitic acid-U-C^{14}

Six specimens of *Nainereis dendritica*, weighting an average of 110 mg each, were preincubated 24 hours in M. B. L. S. W. containing 200 mg streptomycin sulfate per liter. M. B. L. S. W. containing 40 μcuries/l palmitic acid-U-C^{14} (supplied by New England Nuclear) was used as the incubation medium. Homogenization and extraction were according to the standard procedure of Roberts, Abelson, Cowie, Bolton and Britten (1963), which separates the tissue into fractions soluble in cold 5% trichloroacetic acid (TCA), 85% ethanol, ether, hot TCA, and a residual protein precipitate. The ether-soluble fraction was chromatographed along with suitable standards on Merck 250 μ silica gel "G" plates (Brinkmann Instruments, Inc.) developed in chloroform, methanol, water: 65:25:4 (v/v/v). The radioactive spots were located by autoradiography, then scraped into scintillation vials and counted.

Loss of unlabelled oleic acid from the medium

Unless otherwise noted, all organic solvents used in this and the following experiments were Mallinckrodt "nanograde" (glass distilled, nonvolatile matter $< 5 \times 10^{-4}\%$). All glassware was cleaned in chromic acid, coated with Siliclad, rinsed in glass redistilled water and then with hexane. The M. B. L. S. W. was made up from reagent grade salts, glass redistilled water, and was millipore filtered three times before use.

50 specimens of *Stauronereis rudolphi* were cleaned by preincubating them for 24 hours in M. B. L. S. W. to which streptomycin sulfate was added to make a concentration of 200 mg/l. The worms were then rinsed and placed into one of two teflon beakers, each of which contained 50 ml of M. B. L. S. W. in which was dissolved 2.5 μmoles oleic acid per liter. Duplicate 10 ml samples were taken from the control beaker at the beginning of the experiment and from both beakers at the end of one hour. The water samples were acidified with 2 N HCl and extracted 3 times with 2 ml portions of hexane. The extracts were pooled into conical centrifuge tubes, evaporated to dryness under N_2, redissolved in 0.1 ml benzene and quantitatively spotted onto Analtech 250 μ silica gel "G" thin-layer plates which had been divided into 7 mm lanes (method of Downing, 1968). The plates were developed in hexane, ether, acetic acid: 60:40:2 (v/v/v), sprayed with 50% H_2SO_4 (v/v) and heated 20 minutes in a 180° C oven to char the lipid spots. The free fatty acid spots were scanned with a Photovolt Model 520A densitometer using a 396 mμ filter. The product of the peak width at $\frac{1}{2}$ height times the height was a linear function of the weight of oleic acid standard (TLC neutral lipid standard, Sigma Chemical Co.) for values above 2 μgrams.

Analysis of dissolved lipids

Sea water sample "A" was collected in a glass bottle on September 28, 1970, 1 foot below the surface near Berth #158 of Los Angeles Harbor. The 1500 ml sample was put on ice and immediately brought back to the laboratory and filtered with suction through multiple sheets of Whatman #1 filter paper and then through Whatman GF/A glass fiber paper. The filtrate was brought to about pH 2 with 2 N HCl and extracted 3 times by shaking with 150 ml portions of chloroform. The extracts were filtered through chloroform-extracted Whatman 1-PS phase separating paper to remove droplets of water. Two grams of chloroform-extracted Sephadex G-25/40 were added to remove water, salts and non-lipid contaminants according to the method of Williams and Merrilees (1970). After evaporation of the solvent under vacuum, the powder was quantitatively washed onto a sintered glass filter with a little chloroform and the lipid material was eluted with chloroform. The filtrate was evaporated to dryness under a stream of N_2, dissolved in 0.5 ml benzene and stored under N_2 at −20° C. A solvent blank was prepared by treating 500 ml of chloroform in the manner described above for the extract.

Sample "B" was collected on October 1, 1970, at the same location by drawing water from under the algal and mussel mat on floating pilings using a large syringe. The 2 liter sample was treated as the first sample, except that particulates were removed by centrifugation at 0° C in Siliclad coated Nalgene buckets for 25 minutes at 11,600 G (*cf.* Rudolfs and Balmat, 1952).

The chloroform extraction removed more than 99% of palmitic acid-C^{14} dissolved in filtered M.B.L.S.W. Glass fiber filtration removes about half of organic acids dissolved in sea water (Quinn and Meyers, 1971). Recovery of oleic acid-C^{14} added to sample "B" sea water before centrifugation was also about 50%. Virtually no activity was retained by the Sephadex powder after elution with chloroform.

Measured aliquots of the sea water extracts and solvent blank were chromatographed on Analtech thin-layer plates, charred and scanned as above. Peaks were identified and quantified by comparison with Sigma quantitative neutral lipid standards.

The rest of the samples were saponified by heating at 60–80° C for 30 minutes with 1 ml 10% KOH in 50% methanol and the non-saponifiable substances removed by hexane extraction. The mixture was then acidified, 2 ml of water were added, and the fatty acids extracted with hexane. Esterification was carried out by the procedure of Morrison and Smith (1964). After 0.5 ml of benzene and 2 ml of 14% BF_3 in methanol (Applied Sciences Laboratories) were added to the fatty acids, the mixture was heated at 80° C for 15 minutes. 2 ml of water were added to the cooled reaction mixture and the methyl esters extracted 3 times with hexane.

Gas-liquid chromatography (GLC) of the fatty acid methyl esters was done using a Barber-Colman Model 5000 instrument employing a single argon ionization detector. The columns, glass U-tubes 6 feet long and 3.5 mm in inside diameter, were packed with either 10% Apiezon-L on 60/80 mesh Gas Chrom Q or 10% ethylene succinate methylsilicone polymer (EGSS-X) on 100/120 mesh Gas Chrom P (Applied Sciences Laboratories, Inc.). The Apiezon column was operated on a temperature program starting at 175° C and rising to 270° C at the rate of 2° C/minute. Argon gas flow was 60 ml/minute and the injector port and detector were heated to 280° C and 300° C, respectively. The EGSS-X column was operated isothermally at 180° C with carrier gas flowing at 50 ml/minute and injector and detector at 220 and 235° C, respectively. Under these conditions the logarithm of the retention distance is a linear function of the carbon number (James, 1960). Chromatographing the same sample on both columns facilitates identification of the peaks, as the unsaturated esters run ahead of their saturated analogues on the non-polar Apiezon column, and behind the saturated esters on the polar EGSS-X column. The relative weight of each methyl ester was calculated by multiplying the peak height times the retention distance (Brandt and Lands, 1968) on the EGSS-X column and by comparison with K-101 and K-108 FAME standard mixtures supplied by Applied Sciences Laboratories.

TABLE I

Species surveyed for ability to take up dissolved free fatty acids. Unless otherwise noted, the organisms were incubated for 1 hour in M.B.L.S.W. containing a C^{14}-labelled fatty at the concentration specified

Species	Fatty acid	Conc. (µmoles/l)	cpm/g tissue / cpm/g medium	Per cent of medium activity lost	Per cent recovery in organism
Nainereis dendritica	palmitic	0.10	24.6	34	66
	oleic	0.60	56.4	45	66
Stauronereis rudolphi	oleic	0.35	27.4	34	75
Glycera dibranchiata	palmitic	0.14	1.8	18	35
Podarke pugettensis	palmitic	0.08	16.2	28	35
Lumbrineris spp.	palmitic	0.12	4.6	9	42
Cirriformia spirabrancha	palmitic	0.12	4.0	23	49
*Nereis limnicola**	oleic	0.68	6.6	4	86
*Tubifex tubifex***	oleic	0.69	10.6	6	101
	caproic	0.50	12.7	12	95
Urechis caupo	oleic	0.34	3.1	52	92
Amphipholis pugetana	palmitic	0.09	4.4	18	55
Strongylocentrotus purpuratus	palmitic	0.18	3.6	18	45

* Incubated in 0.4% NaCl.

** Incubated in distilled water for ½ hour.

RESULTS

Survey of fatty acid uptake

A number of species of marine worms, the fresh water oligochaete *Tubifex tubifex*, the echiuroid worm *Urechis caupo*, and 2 echinoderms were tested for ability to take up one or more C^{14}-labelled fatty acids. Most were able to concentrate the label at least severalfold over the medium activity (Table I). Radioactivity accumulated in the organism as it was lost from the medium. About 10% of the radioactivity in artificial sea water medium adhered to the surface of the glassware in control beakers containing no organisms. The rest of the radioactivity could be accounted for in the rinse water and mucus thrown off by some of the organisms.

In the first experiments 200 mg of streptomycin sulfate or a mixture of 200 mg streptomycin, 50 mg chloramphenicol, and 500,000 units of penicillin were added to each liter of medium to retard microbial activity. This practice was discontinued when it was found to make no difference in the rate of C^{14} uptake.

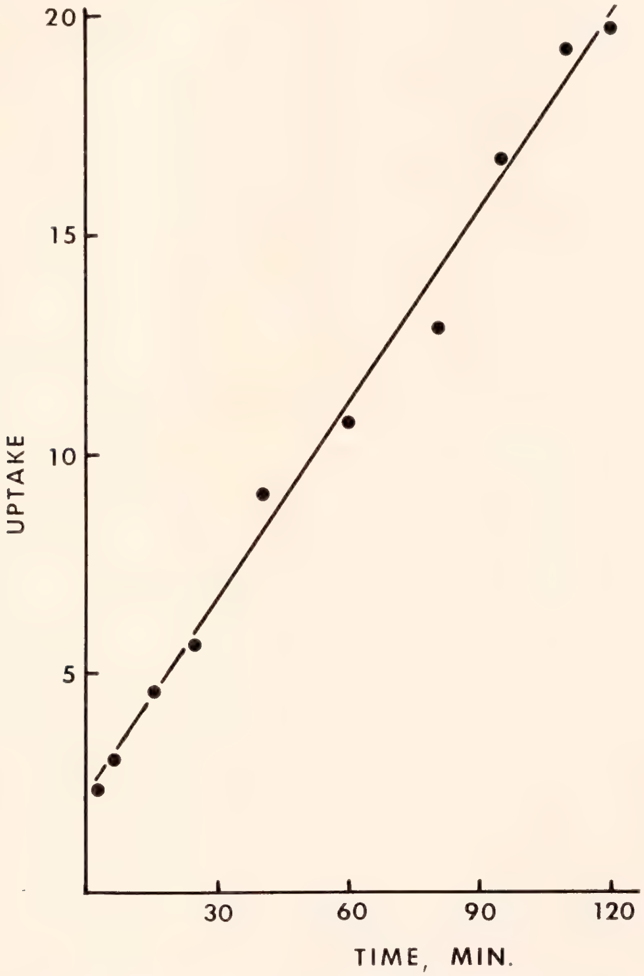

FIGURE 1. Uptake of linoleic acid-1-C^{14} from a 2.0 μM solution—activity per fresh weight of worm tissue (arbitrary units) as a function of incubation time. Each point represents a group of approximately 20 worms. Linear regression lines in this and the following figures were fitted by the method of least squares.

The uptake of C^{14} by live *Nainereis dendritica* exposed to 0.1 μmoles/l of palmitic acid-U-C^{14} was compared to that of individuals that had been killed with KCN. Live specimens were able to concentrate the label by a factor of 25 over the medium activity; dead controls were unable to concentrate the label more than 0.8 times.

The marine worm *Stauronereis rudolphi* was chosen for detailed kinetic studies. When uptake velocities in M. B. L. S. W. were compared to those measured in filtered sea water from L. A. Harbor, there was no difference in the velocity of palmitic acid uptake, but uptake of oleic and caproic acid from sea water was 25% and 50%, respectively, lower than from M. B. L. S. W.

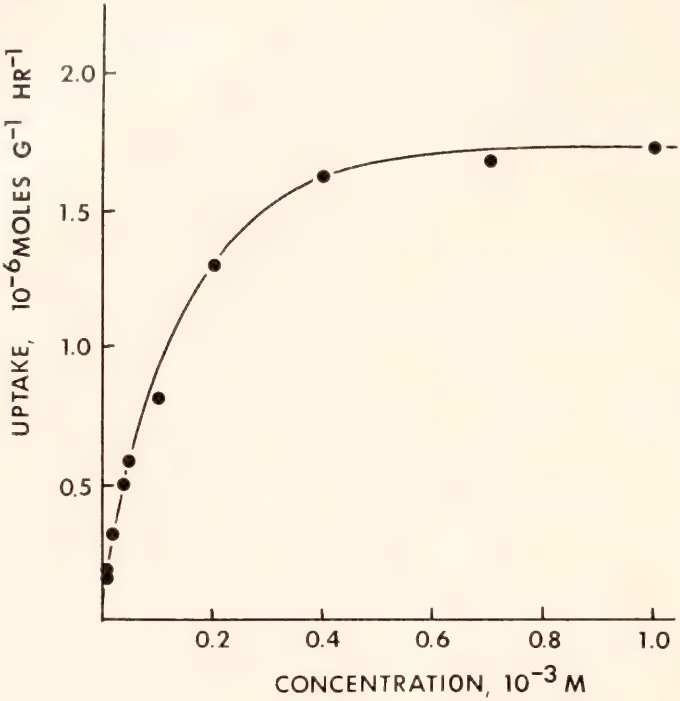

FIGURE 2. Uptake velocity of acetic acid-C^{14} as a function of substrate concentration.

Uptake as a function of body weight

Weight specific uptake of C^{14}-labelled oleic acid by *Stauronereis* decreased with increasing body weight such that the slope of a double logarithmic plot of uptake as a function of weight is 0.80. However, uptake rates in the following experiments were adjusted for variation in worm size simply by dividing the total tissue radioactivity by the fresh weight. The error inherent in this approximation was minimized by using worms of approximately the same size within any given series of experiments.

Uptake as a function of time

Uptake by *Stauronereis* of palmitic, oleic, linoleic, caproic and acetic acid proceeded linearly with time for the 2–3 hour incubation period. In the case of the first 3 acids, there was often an abrupt initial spurt of uptake in the first few minutes (see Fig. 1 for a particularly striking example). In the following

TABLE II

Michaelis-Menten constants for uptake of fatty acids by Stauronereis rudolphi *± 95% confidence intervals. N is the number of groups of worms used for the determination*

Fatty acid	N	K (10^{-6} moles/liter)	V_{max} (10^{-9} moles/g/hour)
palmitic	8	1.09 ± 0.49	20.5 ± 5.4
palmitic	13	0.98 ± 0.38	13.0 ± 3.2
oleic	50	1.26 ± 0.36	41.3 ± 6.5
linoleic	31	18.4	621
caproic	17	12.5 ± 3.6	179 ± 39
acetic	10	102 ± 15	1900 ± 170

kinetic studies the initial spurt was ignored and uptake velocities were calculated after at least 1 hour of incubation to minimize the error.

Uptake as a function of substrate concentration

The graph of uptake velocity as related to ambient concentration of acetic acid (Fig. 2) reveals typical saturation kinetics which can be described by the Michaelis-Menten equation, $v = V_{max} \cdot S/(K_s + S)$, where v is the velocity, S is the substrate concentration, V_{max} equals the maximum velocity and K_s is numerically equivalent to the substrate concentration at which the velocity is one-half the V_{max}.

Uptake of the other fatty acids listed in Table II also follows saturation kinetics except for important deviations found at the higher concentrations of palmitic and oleic acids (Figs. 3 and 4A). When palmitic or oleic acid were introduced into

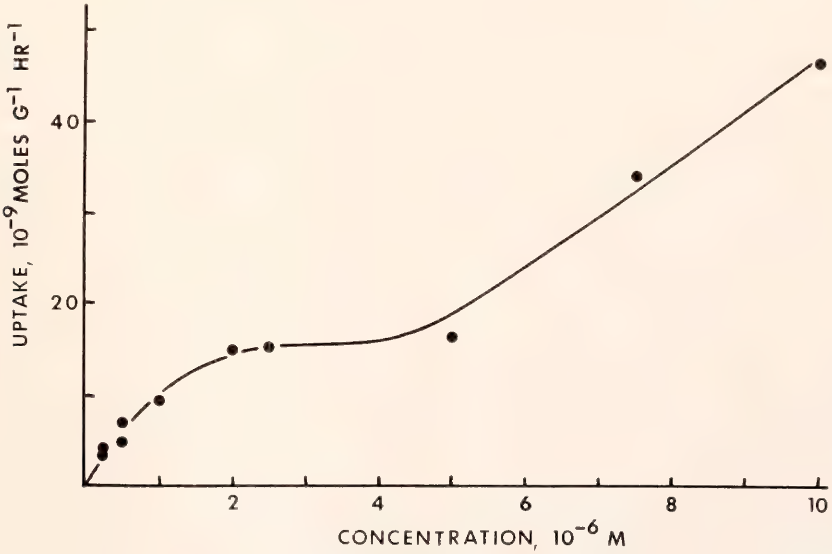

FIGURE 3. Uptake velocity of palmitic acid-1-C^{14} as a function of substrate concentration.

M. B. L. S. W., the solution remained clear up to a concentration of about 5 μmoles/l. Above this concentration a slight opalescence could be detected and further addition of fatty acid resulted in turbidity, which was associated with the anomalously high uptake velocities. Only data from concentrations of 5 μmoles/l

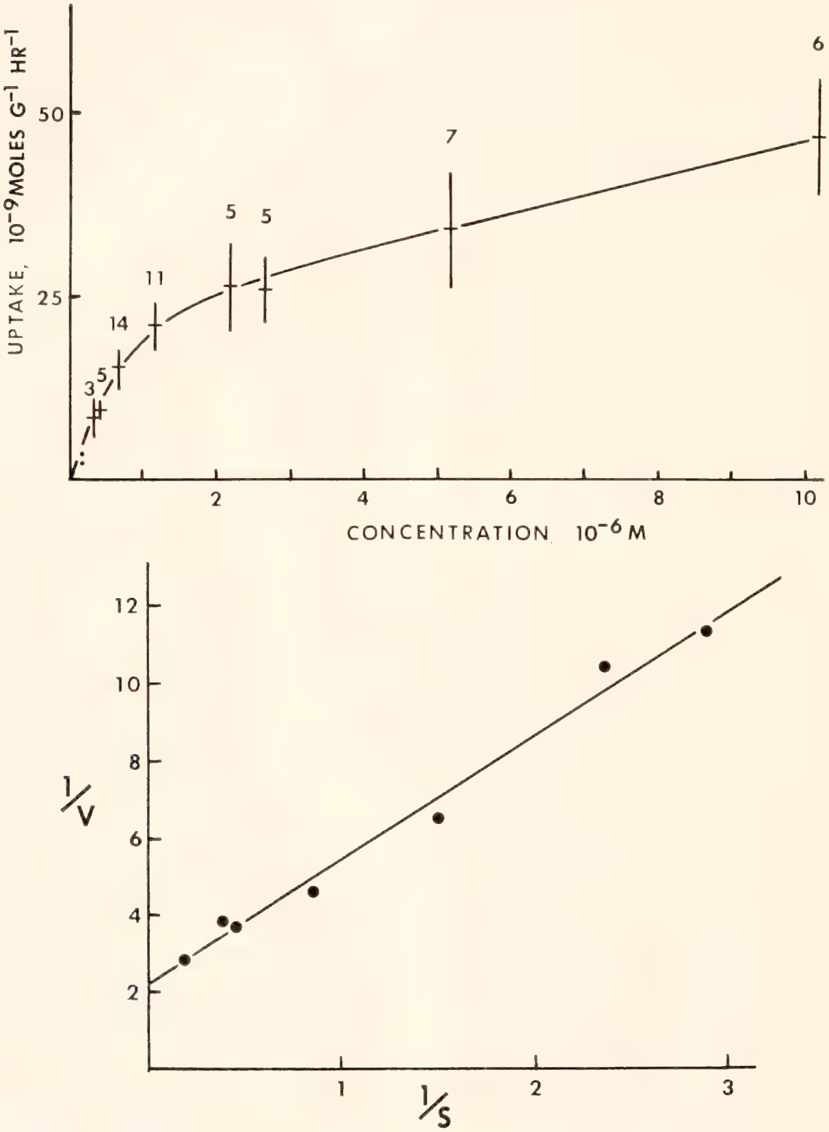

FIGURE 4. Uptake of oleic acid-1-C^{14}; (A.) velocity as related to substrate concentration. The vertical lines delineate the 95% confidence intervals and the numerals are the number of groups of worms used in each determination; (B.) Lineweaver-Burk transformation of the same data (where $V = 10^{-7}$ moles/g/hr and $S = 10^{-6}$ M), omitting the point at $S = 10^{-5}$ M.

and below were used in calculating kinetic parameters of palmitic and oleic acid. The turbidity problem with linoleic and caproic acid was much less severe. Concentrations up to 20 μmoles/l of linoleic acid and 30 μmoles/l of caproic acid could be used without difficulty.

When the reciprocal of the uptake velocity is plotted against the reciprocal of the substrate concentration (Lineweaver-Burk plot), a straight line results in which the y intercept equals $1/V_{max}$ and the slope equals K_s/V_{max}. The Lineweaver-Burk plot of oleic acid uptake is presented here (Fig. 4B) because kinetic data have traditionally been visualized in this manner. However, this method has the disadvantage of disproportionately emphasizing the data at lower substrate concentrations and giving less reliable Michaelis-Menten constants than the Hofstee (v *versus* v/S) or the Woolf (S/v *versus* S) plots of the Michaelis-Menten equation (Dowd and Riggs, 1965). The constants presented in Tables II and III were derived from linear regression lines calculated by the method of least squares for data plotted by one of the last two methods.

TABLE III

Uptake of oleic acid-1-C^{14} inhibited by various fatty acids. Michaelis-Menten constants ± 95% confidence intervals

Inhibitor	N	I (10⁻⁶ moles/l)	K_s (10⁻⁶ moles/l)	V_{max} (10⁻⁹ moles/g/hr)
none	50	—	1.26 ± 0.36	41.3 ± 6.5
linoleic	7	20	4.04 ± 2.02	43.8 ± 16.9
palmitic	16	10	1.89 ± 0.80	44.8 ± 10.7
caproic	20	20	2.73 ± 1.20	50.9 ± 15.1

Data were not adequate to compute Michaelis-Menten constants for stearic acid uptake. Average velocity of 8 groups of worms exposed to 0.48 μmoles stearic acid/l was 0.70×10^{-9} moles/g/hour, about $\frac{1}{5}$ the velocity of palmitic acid uptake at that concentration.

The V_{max} of oleic acid uptake in the presence of constant amounts of linoleic, palmitic or caproic acid was not significantly affected, but the apparent K_s increased (Table III). However, this was significant at the 95% confidence level only for linoleic acid. Oleic acid uptake was not inhibited by a concentration of acetic acid 300 times greater.

Assimilation of palmitic acid-U-C^{14}

After 1 hour incubation 64% of the radioactivity remained in the ether-soluble fraction, 33% appeared in the cold TCA-soluble fraction and 3% in the remaining hot TCA-soluble, alcohol-soluble and protein fractions. The same percentages in the control were 79%, 20%, and 0.2%, respectively. TLC of the ether-soluble fraction revealed that only 28% of the radioactivity remained in the free fatty acid fraction, whereas 98% of the control's radioactivity remained in this fraction (the palmitic acid-C^{14} had been added to the control animals just prior to homogenization in cold 5% TCA). The rest of the radioactivity was incorporated by neutral lipids (48%) and phospholipids (23%).

After the 1 hour incubation, 2 of the worms were rinsed and sealed into flasks containing 20 ml of M. B. L. S. W. At the end of 1 hour they were removed, dropped into scintillation fluid, and the C^{14} activity measured in the liquid scintillation counter. Radioactivity leaked into the medium was measured both immediately and after the medium had been acidified and allowed to stand for 24 hours. Leakage of activity from the worms was only 4.0% and 5.1% of the uptake in 2 trials. 44% and 56% of the activity in the medium disappeared after acidfication of the samples, and was thus considered to be in the form of $C^{14}O_2$.

Loss of unlabeled oleic acid from the medium

The drop in medium concentration of oleic acid in the flask containing *Stauronereis* exceeded that of the control ($P < 0.05$) by 10.4 and 11.0 μgrams oleic acid per 10 ml sample in two separate experiments.

TABLE IV

Quantitative TLC of dissolved sea water lipids. Results are expressed as μgrams carbon per liter sea water ± 1 standard deviation. The values represent the mean of 4 determinations–solvent blank

Rf	Lipid class	μgrams carbon per liter	
		Extract "A"	Extract "B"
0.08	monoglycerides	14.1 ± 1.2	5.8 ± 1.3
0.13	?	8.3 ± 0.5	trace
0.22	sterols	6.0 ± 0.8	5.7 ± 0.8
0.27	diglycerides	—	5.0 ± 1.0
0.31	?	12.9 ± 3.2	20.0 ± 2.7
0.35	free fatty acids	63.7 ± 4.7	51.4 ± 3.6
0.47	?	—	trace
0.52	triglycerides	5.2 ± 0.1	6.0 ± 2.0
0.85	sterol esters, hydrocarbons	—	38.0 ± 1.6
total		110	132

Analysis of dissolved lipids

The neutral lipids extracted from L. A. Harbor sea water are presented in Table IV. In addition to the substances listed in the table, there was a small quantity of material remaining at the origin that migrated in a chloroform, methanol, water: 65 : 25 : 4 solvent system. It included pigments and nitrogen-containing compounds that appeared to be phospholipids, but there was not enough sample left to make positive identifications. Note that about 50% of the neutral lipid material was in the free fatty acid fraction. Assuming an average carbon number of 16, the 58 μgrams of dissolved free fatty acid carbon per liter is equivalent to 0.30 μmoles/l. The solvent blank showed small but measurable amounts of compounds having Rf's that corresponded to free fatty acids, triglycerides, sterol esters and hydrocarbons, which were subtracted from the values measured in the sea water extract before presentation in the table.

Gas chromatography revealed a number of fatty acid methyl esters (Fig. 5 and Table V). The solvent blank contained mostly methyl esters of palmitic, stearic and oleic acid, but the total quantity was only about 8% of that in the extract. The fatty acid compositions of sea water extracts "A" and "B" were so similar that the differences were within the range of experimental error and the two extracts were pooled for the analysis reported.

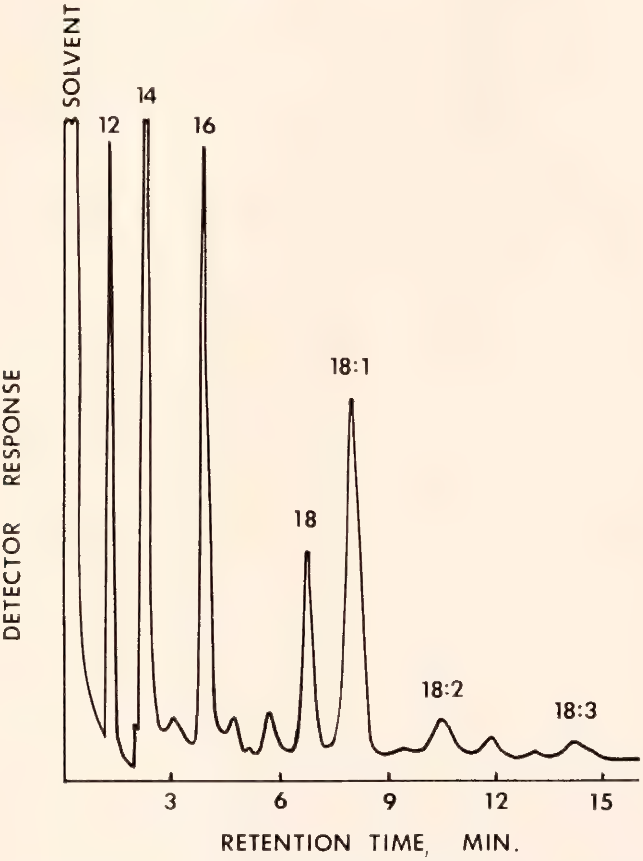

FIGURE 5. Gas-liquid chromatograph on the EGSS-X column of fatty acid methyl esters prepared from sea water extracts "A" and "B." The sudden shift in the tracing between the 12- and 14-carbon peaks represents a 3 times increase in detector sensitivity.

DISCUSSION

Thse studies demonstrate accumulation of dissolved free fatty acids by free-living marine invertebrates. Evidence comes from experiments on (1) the time course of uptake, (2) comparison of uptake rates of dead and live animals, (3) assimilation of labelled fatty acids, (4) net loss of unlabeled fatty acids from the incubation medium.

Uptake of C^{14}-labeled fatty acids proceeds linearly with time for at least 2 to 3 hours. An initial spurt of rapid uptake of long chain fatty acids is probably due to adsorption on the surface of the animals. Vavrečka, Poledne and

Petrásek (1966) found rapid initial uptake of palmitic acid-1-C^{14} into liver slices, which they attributed to surface adsorption. However, the failure of dead animals to accumulate significant amounts of label implies that the long term uptake process is due to some property of the living organism.

Anderson and Stephens (1969) showed that epiflora was responsible for apparent uptake of glycine by marine crustaceans. The same antibiotic mixtures that were effective in eliminating apparent uptake of glycine by crustaceans fail to inhibit fatty acid uptake by marine worms. Therefore it is unlikely that the fatty acid uptake could be attributed to the action of surface bacteria.

TABLE V

GLC on the EGSS-X column of methyl esters of dissolved fatty acids. Per cent composition is by weight. Retention times were measured from the air peak. The chromatogram was continued for 52 minutes, but no peaks appeared after those listed

Carbon number	Retention time minutes	Per cent composition
12	0.89	18.2
14	1.57	15.6
15	2.12	0.5
16	2.75	18.3
16:1	3.32	1.4
17	3.64	0.4
16:2	4.07	2.0
18	4.83	11.2
18:1	5.72	22.8
18:2	7.47	3.3
20	8.47	2.3
?	9.31	0.5
18:3	10.24	2.2
?	11.94	1.3

Analysis of worms exposed to C^{14}-labelled palmitic acid shows that the compound rapidly enters energy and biosynthetic pathways. Pocock, Marsden and Hamilton (1971) found evidence that free fatty acids function as a basic energy substrate in *Nereis virens* and probably have a very high turnover rate, as the animals contained only traces of free fatty acids. Leakage of isotopic label from *Nainereis* back into the medium was slight, but this gives little indication of the true rate of fatty acid leakage, as the identity and specific activity of the leaked compounds are not known.

Extraction and assay of oleic acid containing medium demonstrates that there is net loss of higher free fatty acids from the medium in the presence of *Stauronereis*. Uptake velocities measured by two independent techniques are roughly in agreement. Unlabelled oleic acid disappeared from the medium at the rate of 37.9×10^{-9} moles/g/hour. The velocity calculated from the rate of oleic acid-C^{14} accumulation at the same concentration is 26×10^{-9} moles/g/hour.

Uptake appears to occur across the body wall rather than the gut. A dozen specimens of *Stauronereis rudolphi* weighing a total of 300 mg can easily remove a third or more of the radioactivity in 50 ml of M. B. L. S. W. in an hour.

This would imply a rate of water movement through the gut for which there is no evidence. They were never observed to pump water through the gut. The dark gut contents, which can be seen against a light, take a matter of hours or days to pass out after the worms have been put into clean water. The slope of the double logarithmic plot of uptake as a function of weight, which is less than one, indicates that we may be dealing with a property related to the surface of the organism (*cf.* Reish and Stephens, 1969).

The uptake mechanism of oleic and palmitic acid appears to have two components. However, attempts to apply a "diffusion" correction to the data resulted in distortions of the data at lower concentrations, which suggested that the second component operates only at the higher concentrations. The second component correlates with the appearance of turbidity in the medium, which makes more likely an interpretation relating the anomalously high uptake velocities with a physical change of the fatty acid solution rather than attributing them to a property of the membrane. Palmitic acid, which has the most marked increase of uptake rate, is the least soluble fatty acid used in the kinetic experiments. The phenomenon is absent in the case of caproic and acetic acids, which are both very soluble. Chappell, Arme, and Read (1969) found evidence that palmitate uptake in the tapeworm *Hymenolepis diminuta* is enhanced by the presence of molecular aggregates. Bailey and Fairbairn (1968) noted more rapid uptake of oleic acid by *Hymenolepis* from micellar solution. Hoffman (1970) compared uptake of oleic acid-C^{14} by everted sacs of rat jejunum above and below the critical micelle concentrations of 2 detergents, one a bile salt and the other a high molecular weight non-ionic detergent. Uptake was greater from micellar media of both detergents, indicating an effect of micellar phase *per se* in promoting uptake, possibly by serving as a pool to maintain the concentration gradient of fatty acid at the transport surface. He also demonstrated that uptake is by single molecules rather than as intact micelles. A similar mechanism with respect to palmitic and oleic acid uptake in *Stauronereis* could account for the present data.

Southward and Southward (1970) also encountered anomalously high uptake of palimitic acid by the pogonophore *Siboglinum ekmani* at the same concentrations that I did with *Stauronereis*. When they eliminated the anomalous points they found a double logarithmic relationship of uptake velocity and palmitic acid concentration from 5×10^{-9} M to 6×10^{-6} M. The authors suggested pinocytosis as a possible uptake mechanism. They measured uptake velocities of 10^{-8} moles/g/hour from a 10^{-6} M palmitic acid solution, which falls within the range of velocities that I measured for palmitic acid uptake by *Stauronereis* at that concentration. Sodium butyrate was taken up much more slowly by *Siboglinum atlanticum* ($V_{max} = 2.7 \times 10^{-9}$ moles/g/hour) and had a much lower K_s (3×10^{-9} M) than uptake of short chain acids by *Stauronereis,* yet they found saturation of the uptake mechanism at higher concentrations.

V_{max}'s vary and tend to decline as the condition of the worm cultures deteriorates, but K_s's remain relatively constant. Two separate determinations of the Michaelis-Menten constants for uptake of palmitic acid are given in Table II as a typical illustration of the phenomenon. This is compatible with a model that attributes uptake to a mediated transport system having a finite number of active sites on the surface of the organism. The affinity of a given site for the substrate, which is expressed as the K_s, is a constant property of the transport molecule, whereas the V_{max} varies with the number of functioning sites, which is inconstant.

The increase of apparent K_s for oleic acid uptake in the presence of linoleic, palmitic, or caproic acid while the V_{max} remains unchanged shows that the inhibition is competitive. This implies a common transport pathway for these fatty acids. The failure of acetic acid to inhibit oleic uptake suggests a separate site for uptake of acetate.

The dissolved lipid composition of the sea water samples is unusual in that such a large percentage consists of free fatty acids. Lela Jeffrey of Texas A&M University (personal communication) considered that only about 1% of the lipid substances passing a 0.45 μ filter are free fatty acids. Garrett (1967) reported 1–25% free fatty acids in lipids found in surface slicks. The recovery of free acids may depend to a large degree on the method used in handling the samples. The silicone treatment of all glassware used in handling my sea water samples may have increased recoveries of the free acids, as fatty acids have a tendency to stick to untreated glass surfaces (Green, 1969). Quinn and Meyers (1971) reported that recovery of heptadecanoic acid from sea water after passage through Whatman 54, Whatman GF/C glass fiber or Millipore HA membrane filters was 98.5, 51.9 and 14.2 per cent, respectively. Millipore membrane filters have been used by nearly all workers reporting dissolved lipids. Jeffrey used glass fiber paper only in the work reported in her 1970 paper, and Garrett (1967, page 223) did not filter his samples "to avoid losses of surface-active material due to selective adsorption onto the filter." Max Blumer of the Woods Hole Oceanographic Institute (1970) reported 19.7 μg/l total fatty acids in Buzzards Bay, Mass. sea water that he filtered through Whatman 54 paper. He estimated from IR spectra (personal communication) that roughly one-half was in the free acid form.

A puzzling feature of the fatty acid spectrum is the absence of the higher polyunsaturated fatty acids that are characteristic of marine lipids. Jeffrey (1966) reported dissolved fatty acids having up to 22 carbon atoms and as many as 6 double bonds in the longer acids. It is possible that much of the dissolved lipid material in the upper reaches of the harbor comes from leaching of sediments, fresh water runoff and organic pollution. Palmitic acid is almost always the most abundant fatty acid in marine sediments, which are also characterized by the virtual absence of polyunsaturates (Parker, 1967). Peterson (1967) found that the 14-, 16-, and 18-carbon acids dominated the sedimentary fatty acids and 16:1 and 18:1 were the dominant unsaturated acids. The dissolved fatty acids in L. A. Harbor exhibit a similar composition (Table V).

Hunter and Heukelekian (1965) found that the soluble organic matter in domestic sewage was mostly ether soluble, of which 56% was lower organic acids. Sewage pollution could explain the presence of C_{12} and C_{14} fatty acids in amounts approaching palmitic acid. My methods of extraction and analysis would not detect organic acids of less than 12 carbon atoms, but their presence might explain the 50% inhibition by harbor sea water of caproic acid-C^{14} uptake.

Although V_{max}'s of fatty acid uptake by *Stauronereis* are very low, K_s's are also low, allowing transport to take place efficiently from substrate concentrations in the submicromolar range. The V_{max} and K_s of palmitic acid uptake are about

3 orders of magnitude lower than reported for the intestinal parasite *Hymenolepis diminuta* by Chappell, Arme, and Read (1969). This suggests an adaptation to the low environmental concentrations encountered by the free-living worm. As a nutritional source, however, the process makes only a small contribution to the metabolic needs of the organism. Assuming a QO_2 of 0.05 ml O_2/gram/hour, the percentage of oxygen uptake that could be supported by accumulation of free fatty acids by *Stauronereis* at the concentrations measured in L. A. Harbor sea water is: palmitic acid, 2.0%; oleic acid, 4.5%; linoleic acid, 0.8%. If one assumes that the other fatty acids are taken up in proportionate amounts, uptake of total long-chain fatty acids could support 16% of the worm's oxidative metabolism. It should be noted, however, that the actual nutritional needs of the organism are likely to be considerably greater than that necessary to support the oxygen consumption in order to allow for growth, gamete production and anaerobic metabolism (see Stephens, 1963). The contribution would be greater in areas having higher fatty acid concentrations. To my knowledge, there have been no other published quantitative analyses of dissolved free fatty acids in inshore waters or recent marine sediments.

Several possibilities other than a reduced carbon source suggest themselves. Uptake could be a source of specific fatty acids, phospholipids, or a route for entry of pheromones with fatty acid moities. It is tempting to speculate about possible uptake of synthetic soaps, detergents, pesticides and other man-made lipid substances, although these substances have not yet been investigated.

I would like to acknowledge the guidance of Grover C. Stephens in the research and writing.

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SUMMARY

1. It has been established that representative marine annelids can accumulate and metabolize dissolved free fatty acids.

2. Net uptake of fatty acid from dilute solution by the polychaete *Stauronereis rudolphi* was confirmed by the loss of total higher fatty acids from the medium.

3. Uptake takes place across the body wall by a mediated transport system having kinetic properties analagous to those of enzyme-catalyzed reactions, including saturation at high substrate concentrations and competitive inhibition.

4. At concentrations above 5 μmoles/l, palmitic and oleic acids are taken up at anomalously high rates probably due to the formation of micelles or molecular aggregates.

5. Dissolved free fatty acids occurring in Los Angeles Harbor sea water were extracted and analyzed by thin-layer and gas chromatography.

6. Although the K_s's of higher fatty acid transport are low, allowing efficient uptake from low concentrations, the levels of free fatty acids found in the natural

environment are probably too low for uptake to support more than a few per cent of the organisms's metabolism.

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EFFECT OF TEMPERATURE ON THE METABOLIC RATE OF SEA URCHINS[1]

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There are several species of sea urchins along the Oregon coast which occupy overlapping but limited vertical ranges. According to Mortensen (1943) the fragile urchin, *Allocentrotus fragilis*, has a bathymetric distribution of about 50–1150 m. Both *Strongylocentrotus franciscanus*, the red urchin, and *Strongylocentrotus purpuratus*, the purple urchin, occur intertidally as well as subtidally. The purple urchin is abundant in the intertidal but also has been dredged from depths as great as 64 m. The distribution of the red urchin is more subtidal than that of the purple urchin and extends from the low tide line to depths of at least 125 m (Mortensen, 1943; Swan, 1953; McCauley and Carey, 1967; Ricketts, Calvin, and Hedgpeth, 1968). The more intertidal species would presumably be exposed to greater thermal variation and show different patterns of metabolic compensation for temperature, compared to subtidal forms.

Although the effect of a variety of factors on the metabolism of sea urchins has been studied—*e.g.*, body size (McPherson, 1968), nutritional state (Farmanfarmaian, 1966), reproductive state (Giese, Farmanfarmaian, Hilden, and Doezema, 1966), oxygen tension (Johansen and Vadas, 1967), salinity (Giese and Farmanfarmaian, 1963), and burrowing (Lewis, 1968)—there are relatively few reports concerning the effect of temperature. Farmanfarmaian and Giese (1963) investigated the metabolic rate-temperature (R-T) relationship in *Strongylocentrotus purpuratus* following temperature acclimation and reported the usual higher metabolic rates in the cold-acclimated forms. In the sea urchin, *Eucidaris tribuloides*, McPherson (1968) has observed higher metabolic rates in winter-acclimatized forms than those of summer at the corresponding temperatures. In this study we examine the metabolic rate-temperature relationships (R-T curves) for summer-acclimatized *S. purpuratus*, *S. franciscanus*, and *A. fragilis*.

METHODS AND MATERIALS

Definitions and protocols

The terms acclimatization and acclimation are used in this paper according to the definitions of Prosser and Brown (1961). Acclimatization is considered as a nautrally occurring physiological compensation to multiple changes in the environment. Acclimation is considered as a compensatory physiological change

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resulting from exposure in the laboratory to a single factor, with other environmental factors maintained at as constant a level as possible.

Metabolic rate determinations were carried out over a range of temperatures on animals kept in laboratory tanks for short periods (generally no more than 2–3 days) after collection. These urchins are thus "field-acclimatized."

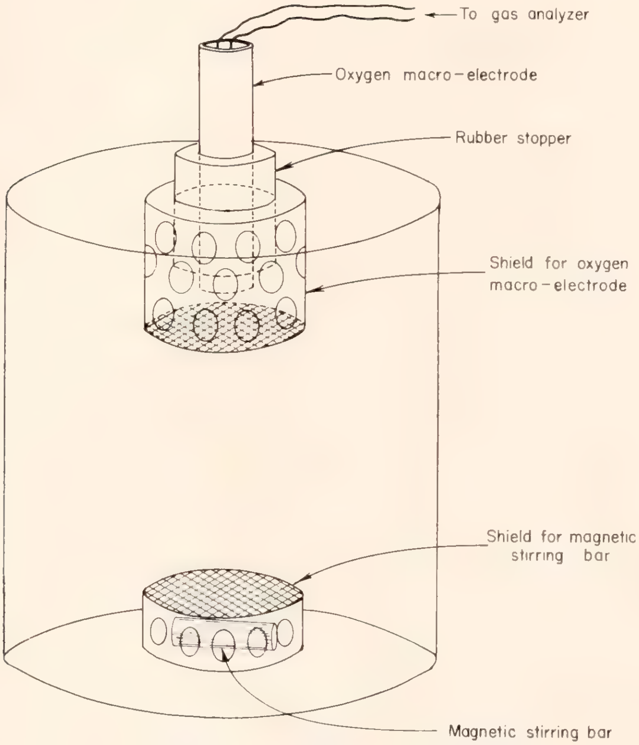
To gas analyzer
Oxygen macro-electrode
Rubber stopper
Shield for oxygen macro-electrode
Shield for magnetic stirring bar
Magnetic stirring bar

FIGURE 1. Respiratory container.

Collection and maintenance of animals

Purple urchins and red urchins were collected from several different locations on the Oregon coast, over a latitudinal range of 1° 30′. Fragile urchins were dredged with an otter trawl from depths of 150 and 200 m along the oceanographic station line extending directly outwards from Newport, Oregon. Urchins were maintained in aerated, running sea water in fiber-glass holding tanks.

Measurement of oxygen consumption

Oxygen consumption was determined by measuring changes in the oxygen tension of filtered sea water in which the urchin was submerged. The urchin was placed in a closed and sealed "Tupperware" plastic container of suitable size. A variety of sizes was necessary owing to the size ranges of the urchins tested (64.6–177.6 g, 207.7–1658.7 g, and 42.7–115.6 g for the purple, red, and fragile urchins, respectively). Resulting sea water volumes ranged from 1.72 to 9.20 liters. A magnetic stirring bar was placed at the bottom of each container, and a slow stirring rate, created by an immersible magnetic stirrer (Tri-R Instrument Co.) was used in all studies to provide for uniform temperature and oxygen tension values. A Beckman oxygen macro-electrode was used in conjunction with a physiological gas analyzer (Beckman Spinco model 160) to determine the oxygen tension. Appropriate shields restrained the urchins from interfering with the operation of the stirring bar and the oxygen macro-electrode. The arrangement is pictured in Figure 1.

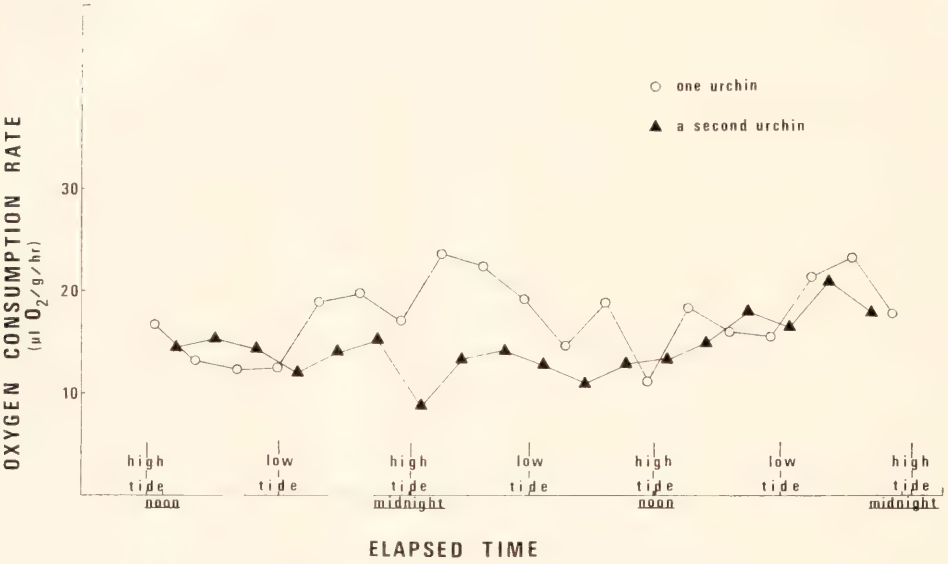

FIGURE 2. Relationship between oxygen consumption rate and elapsed time for *Strongylocentrotus purpuratus*.

The temperature range used was 6° to 24°, with measurements made at 3° intervals. Temperature was maintained with a Bronwill thermoregulator combined with a portable cooling unit (Precision Scientific Company). Urchins were placed in the containers about 5 hrs for equilibration in aerated sea water at 6°, the initial test temperature. The container was sealed and oxygen tension recorded at 10–20 min intervals. In this manner from 3 to 6 measurements of oxygen uptake were obtained. The temperature was then changed to the next level while the respiratory container was aerated. After reaching the new temperature, a one hour period of equilibration was allowed before measurements of oxygen uptake were made. Oxygen tension of the sea water in the container at no time fell below 75 mm Hg, and runs at any one temperature were usually terminated at considerably higher oxygen levels. Measurements of chamber water pH were made from samples taken before urchins were placed in the container, and at the conclusion of testing. Declines of 0.1–0.2 pH unit were observed for initial experiments with urchins. The practice was not continued for the remaining experiments. No effort was made to prevent fouling although no indication of it was observed. All rates are expressed as microliters of oxygen consumed per gram wet weight per hour ($\mu l\ O_2/g/hr$).

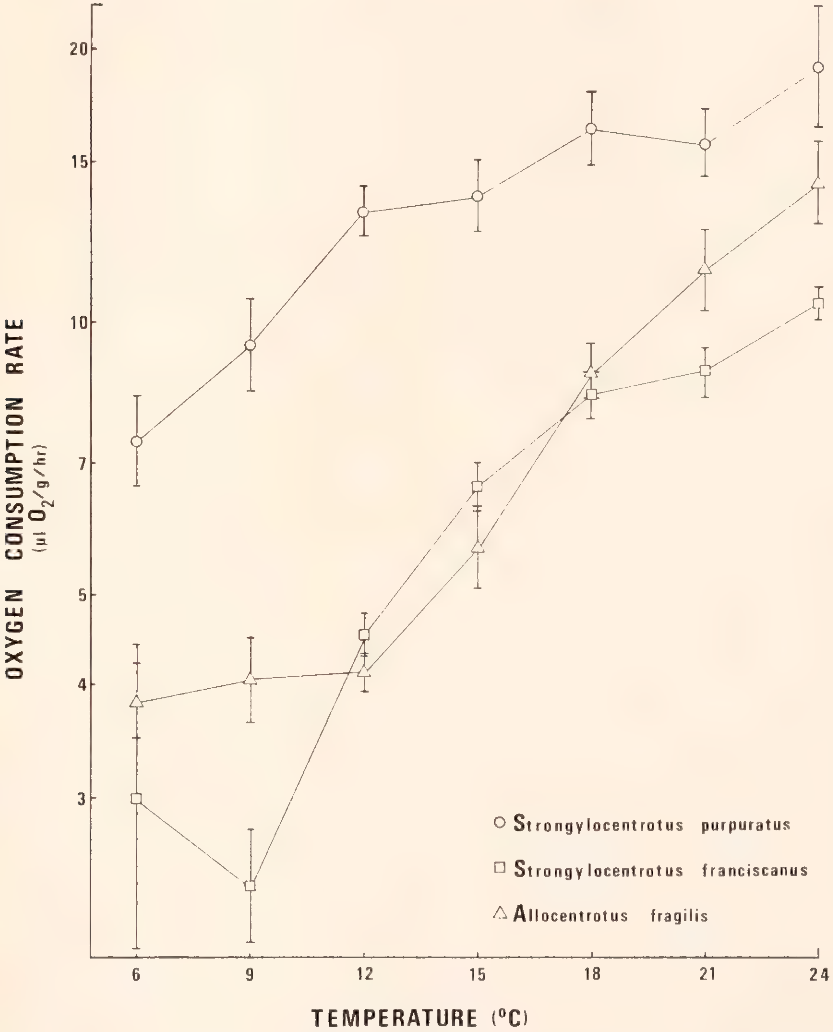

FIGURE 3. Oxygen consumption rate-temperature curves for field-acclimatized sea urchins. Vertical bars indicate 95% confidence limits.

RESULTS

In a preliminary series of tests, the oxygen consumption rates of several individual urchins were determined at frequent intervals for periods greatly exceeding the length of the metabolic rate-temperature experiments. All tests were run at a temperature of 12°. Figure 2, showing the results of a 36 hour experiment—

using two purple urchins concurrently, is representative of the results obtained. There appears to be no clear indication of rhythms in metabolic rate associated with a particular time of day or tidal cycle.

The effect of size upon metabolism is well documented (Zeuthen, 1947, 1953; Hemmingsen, 1960; Prosser and Brown, 1961). McPherson (1968) describes an inverse relationship between metabolic rate and size for the tropical urchin *Eucidaris tribuloides.* Farmanfarmaian (1966) in a review of echinoderm respiratory physiology reports decreasing levels of metabolism with size. In the present study metabolic rates for the purple, red, and fragile urchins are expressed as μl O_2/g/hr corrected to correspond to 97.0, 360.0, and 70.0 g (wet weight) animals, respectively, which constitute approximate mean weights of the test animals. Rates were calculated by determining the linear regression equation for the relation of body size to metabolic rate for all animals under a given set of conditions (species and test temperature). Observation number (n) was 39–42, 22–41, and 13–18 for 12 purple, 12 red, and 6 fragile urchins, respectively. The size-corrected rates are then plotted against temperature in the form of semilog plots (Fig. 3). Vertical bars indicate interval estimates of the population means with 0.95 confidence limits and derive from the following expression (Snedecor and Cochran, 1967):

$$\mu = \hat{Y} \pm t_{0.05} S_{\hat{Y}}$$

where

$$S_{\hat{Y}} = S_{y.x} \sqrt{\left(\frac{1}{N}\right) + \left(\frac{x^2}{\sum x^2}\right)}$$

$$\text{d.f.} = N - 2$$

Figure 3 depicts the metabolic rate-temperature relationships for summer field-acclimatized purple, red, and fragile urchins. Q_{10} values for all temperature intervals are presented in Table I. Rates of the purple urchin rise with temperature from 6° to 12° and 21° to 24° but show considerable rate-temperature independence (Q_{10}'s 0.883 to 1.74) from 12° to 21°. Metabolic rate of the red urchin rises with temperature from 9° to 24°. The mean rate at 6° is higher than at 9°. However, the variability at 6° is greater than at any temperature tested. The rates at both 6° and 9°, moreover, are so low as to be almost

TABLE I

Q_{10} values for oxygen consumption rate-temperature relationships

Temperature range	*Strongylocentrotus purpuratus*	*Strongylocentrotus franciscanus*	*Allocentrotus fragilis*
6°–9°	2.25	0.483	1.22
9°–12°	3.10	8.26	1.07
12°–15°	1.16	3.45	2.82
15°–18°	1.74	2.18	4.47
18°–21°	0.883	1.19	2.35
21°–24°	1.88	1.86	2.11

undetectable. In the fragile urchin oxygen consumption rate appears temperature independent between 6° and 12° and temperature dependent from 12° to 24°.

Other than temperature and body size, factors contributing to variability include availability of oxygen, activity, the nutritional state, and the reproductive state of the animal. In an attempt to minimize the possible influence of reproductive condition (Giese, Farmanfarmaian, Hilden, and Doezema, 1966) all experiments were performed in summer and early fall. At this time the gonads of the intertidal urchins are said to be spent and/or in a state of incipient gametogenesis (Bennett and Giese, 1955; Holland and Giese, 1965; Chatlynne, 1969). In regard to the relation between metabolic rate and oxygen tension, the earlier literature refers to echinoids in general as "conformers" (Hyman, 1929; Farmanfarmaian, 1959, 1966; Prosser and Brown, 1961). Steen (1965) describes a constant level of oxygen consumption for *Strongylocentrotus droebachiensis* at 6° but conformity at 16°. Johansen and Vadas (1967) studied several species of strongylocentrotids and found that oxygen consumption was independent of the ambient oxygen tension down to 60–70 mm Hg, although a close correlation between the oxygen consumption rate and the oxygen tension of the coelomic fluid was noted. In the present study oxygen tension in the respiratory containers never fell below 75 mm Hg.

DISCUSSION

Of the three species used in the study, the purple urchin, *S. purpuratus*, occupies the most exposed habitat and would be expected to encounter considerable fluctuation in temperature during tidal cycles. Gonor (1968) reports a range of 8.5° in the temperature of the surf at Agate Beach during August. Moreover, Gonor (1968) observes that during summer low tides, which occur during daylight hours, the mean internal temperature of purple urchins may rise to as much as 10° above that of the sea surface. Metabolic rate of *S. purpuratus* is essentially independent of temperature between 12° and 21° (Fig. 3). We suggest that the wide temperature range over which rate of oxygen consumption is relatively temperature independent may be adaptive in permitting these animals to maintain constant metabolic rates in the face of considerable temperature variation.

A number of recent studies have reported low Q_{10}'s for a variety of rate functions in many intertidal invertebrates (Baldwin, 1968; Newell and Pye, 1970a, 1970b, 1971). The temperature independence of metabolic rate between 12° and 21° shown by purple urchins in the present study is consistent with these findings. Metabolic rate is temperature dependent below 12° and above 21°.

Although red urchins are found in the lowest reaches of the intertidal habitat, they are for the most part subtidal forms (Mortensen, 1943; Swan, 1953; McCauley and Carey, 1967; Ricketts, Calvin, and Hedgpeth, 1968). Q_{10}'s for metabolic rate of *S. franciscanus* are high at all parts of the test temperature range excepting 6°–9° (Table I). From 9° to 12°, a range to which this species might reasonably be exposed in nature, an extremely high Q_{10} of 8.26 was found. It would appear that *S. franciscanus*, in contrast to the more intertidal *S. purpuratus*, has a metabolic rate which is essentially temperature dependent.

The fragile urchin, *A. fragilis,* is a deep-water benthic form exposed to a constant low temperature. The annual average bottom temperature measurements from January 1967 to January 1969 at the two stations where the fragile urchin was dredged were 8.20° ± 0.802 and 7.58° ± 0.588 (A. G. Carey, Jr., Oregon State University, personal communication). The rate-temperature curve for fragile urchins (Fig. 3) shows Q_{10}'s very close to 1.0 from 6° to 12° (Table I). From 12° to 24° Q_{10}'s are 2.0 or greater, illustrating temperature dependence of metabolic rate. Thus, within the narrow temperature limits to which *A. fragilis* might normally be exposed, it would seem that metabolic rate is maintained at a constant level. It is noteworthy that fragile urchins have much lower rates of oxygen consumption at all test temperatures, compared to purple urchins, even though adults of the two species are similar in size. This may reflect differences in nutrition and/or overall activity of the two species. Paine and Vadas (1969) acknowledge the essentially herbivorous nature of *S. purpuratus,* whereas McCauley and Carey (1967) suggest a detrital form of nutrition for *A. fragilis.*

All three species were tested in summer and early fall, and it is possible that different rate-temperature curves would be found at other times of the year. In the urchin *Eucidaris tribuloides,* for example, McPherson (1968) has observed higher metabolic rates at corresponding temperatures in winter-acclimatized animals compared to summer forms.

The authors gratefully acknowledge assistance from Dr. Andrew G. Carey, Jr., who provided facilities aboard the research vessel YAQUINA for collecting *A. fragilis.*

SUMMARY

1. The metabolic rate-temperature relationship for several species of sea urchins from different habitats has been determined. Rates were determined over a range of temperatures on animals kept in the laboratory for short periods of time ("field-acclimatized" animals).

2. The benthic *Allocentrotus fragilis* showed rate-temperature independence from 6° to 12°, a range including the habitat temperatures, but dependence (high Q_{10}'s) from 12° to 24°.

3. The relatively subtidal *Strongylocentrotus franciscanus* showed little rate-temperature independence.

4. The more exposed *Strongylocentrotus purpuratus* showed rate-temperature independence over a wide range of temperature (12°–21°).

5. The metabolic rate-temperature results for the field-acclimatized urchins appear to be consistent with the temperature fluctuations of their respective habitats.

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SOME FACTORS AFFECTING VERTICAL DISTRIBUTION AND RESISTANCE TO DESICCATION IN THE LIMPET, *ACMAEA TESTUDINALIS* (MÜLLER)[1]

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The vertical distribution of many organisms in the intertidal zone is governed by their ability to tolerate the stresses of exposure (Stephenson and Stephenson, 1949; Wilbur and Yonge, 1964). Desiccation, one of these stresses, is a dominant factor influencing distribution (Stephenson, 1942). An organism inhabiting higher levels of the intertidal zone must tolerate longer periods of desiccation than an organism inhabiting lower levels.

Broekhuysen (1940) investigated the role of desiccation in controlling the vertical distribution of six species of intertidal gastropods of the genera *Littorina*, *Thais*, *Oxystele*, and *Cominella*. Results of his laboratory experiments indicated that species inhabiting higher levels tolerated greater body water loss before death than species inhabiting lower levels. He concluded that species from higher levels were least sensitive to desiccation. Davies (1969) studied limits of tolerance to desiccation in the limpet *Patella vulgata*. He found that high intertidal specimens of *P. vulgata* were tolerant of a greater loss of body water than low intertidal specimens. He concluded that, within a species, specimens from higher levels were more tolerant of desiccation.

Other investigators have shown the same interspecific and intraspecific variation in tolerance of desiccation between organisms found high and low in the intertidal zone (Colgan, 1910; Gowanloch and Hayes, 1926; Allanson, 1958; Brown, 1960; Davies, 1965).

Intertidal organisms exhibiting a high tolerance of desiccation possess behavioral, physiological or morphological characteristics which are adaptive. In limpets, size and shape of the shell are characteristics which have been correlated with tolerance of desiccation. In *P. vulgata*, Orton (1932) observed that steeper shells were predominant among those from lower levels. Davies (1969) observed the same phenomenon in *P. vulgata*. He stated that steeper shells were adaptive because the smaller circumference per unit volume reduced the amount of water lost between the edge of the shell and the substrate.

Body size has also been related to tolerance of desiccation in limpets. Lewis (1954), Frank (1965), Glynn (1965), and Blackmore (1969) found that large limpets were most numerous at upper levels of the intertidal zone and small limpets were most numerous at lower levels. Davies (1969) determined that rate of body water loss varied inversely with size in *P. vulgata*. Larger limpets could inhabit upper intertidal levels because they lost body water less rapidly. Shotwell (1950) however, found smaller limpets more numerous in the upper intertidal levels, with large individuals restricted to lower levels. He attributed this to relatively greater storage space for water between the shell and mantle in small limpets.

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The size distribution of limpets in the intertidal zone is not always correlated with tolerance of desiccation. Sutherland's (1970) study of the population dynamics of *Acmaea scabra* showed that lower population density of limpets in the upper zone resulted in a more rapid growth rate and a population of larger limpets. In the lower zone, however, high population density restricted growth and the limpets were smaller. In addition to promoting rapid growth, the lower density of the upper zone resulted in a lower mortality rate than the lower zone.

The vertical distribution and its relation to tolerance of desiccation in the New England limpet, *Acmaea testudinalis* has never been reported in the literature. This species, which ranges from Labrador to Connecticut, inhabits the rocky substrata of exposed shores (Miner, 1950; Russell Hunter and Brown, 1964). I have observed that its vertical distribution, in Maine, extends from 1.0–1.5 m above mean low water, into the subtidal area.

The purpose of this study was to determine if *A. testudinalis,* like other limpets, shows intraspecific differences in tolerance of desiccation, those inhabiting the upper levels of the vertical range presumably being most tolerant of desiccation, and those from the subtidal area least tolerant, and whether limpets which are most tolerant exhibit any significant differences in shell shape or size.

MATERIALS AND METHODS

Limpets from three contrasting habitats were compared in this study: (1) a vertical rock face group (VRFG), (2) a tide pool group (TPG), and (3) a subtidal group (SG). Specimens were collected from two intertidal habitats and one subtidal area along the west shore of Schoodic Peninsula, Hancock County, Maine, (44° 22′N, 68° 04′W) on September 20 and October 16, 1969. Limpets collected from vertical rock faces were found approximately 0.5–1.5 m above mean low water. Specimens collected from tide pools were found near mean low water level, and those obtained subtidally were found approximately 2.0–4.0 m below mean low water.

Collections from vertical rock faces and tide pools were made at low tide by wading, and from the subtidal habitat by SCUBA diving. Approximately 360 specimens were collected from each habitat, each day, except that only 270 specimens could be obtained from the tide pools on October 16. Individuals were removed from the substrate by inserting a thin spatula blade under the foot and prying gently. Individuals with damaged shells were discarded. Specimens were placed in plastic bags containing sea water, placed on ice, and taken to the laboratory within 12 hours.

Two laboratory experiments were conducted in which limpets from each habitat were subjected to periods of desiccation. The specimens collected on September 20 were used in experiment (1). Those collected on October 16 were used in experiment (2) which was identical to experiment (1) except for differences in the number of specimens used, as noted below. A total of 360 specimens from each habitat was placed, 20 specimens per dish, in 18 dry, plastic petri dishes, 14.0 cm in diameter. As a control, 60 specimens from each habitat were covered with artificial sea water at 15° C (the sea water temperature at the collecting site on September 20). The sea water was changed every 24 hours. Five groups, each containing 60 specimens from each habitat, were left uncovered and subjected to desiccation at temperatures ranging from 19.5 to 21° C, the seasonal air temperature. Because only 270 specimens were obtained from the tide pools on October 16, a control group and five other groups, each containing 45 limpets, were used in experiment (2). Fifteen specimens were placed in each petri dish.

TABLE I

Number and per cent of limpets from vertical rock face, tide pool and subtidal habitats, surviving desiccation at six time periods

Time in hours	Vertical rock face				Tide pool				Subtidal			
	Experiment 1		Experiment 2		Experiment 1		Experiment 2		Experiment 1		Experiment 2	
	Number of 60	%	Number of 60	%	Number of 60	%	Number of 45	%	Number of 60	%	Number of 60	%
0	60	100	39*	98	59	98	45	100	60	100	60	100
12	57	95	60	100	51	85	41	91	60	100	54	90
24	33	55	49	82	7	12	11	24	27	45	14	23
36	8	13	10	17	1	2	0	0	2	3	4	7
48	1	2	4	7	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0	0	0

* Number surviving of 40.

Each of the five groups was desiccated for a specific time period: 12, 24, 36, 48, or 60 hours. At the end of the desiccation period, each group was covered with 15° C sea water and refrigerated at 15° C for 24 hours, after which it was examined for survivors. The control group was examined at the same time as the group which had been desiccated for 60 hours. The criterion for determining survival was movement of the foot in response to mechanical stimulation. This method was used successfully by Brown (1960) for six species of gastropods.

In experiment 2, five dead specimens were counted in one of the three petri dishes from the VRF control group. The high mortality in this dish was attributed to a build-up of fecal matter around the specimens. This occurred because of a failure to change the sea water in this particular dish for 48 hours. Data obtained from this dish were omitted from the results.

Per cent survival data from the two desiccation experiments were analyzed statistically using the IBM 360 computer at the University of Maine Computing Center in Orono. A randomized block design analysis of variance and a Duncan's multiple range test ($P = 0.05$) were used to determine if survival decreased significantly with increased desiccation time. The two analyses described above were also used to determine whether limpets from one habitat group survived better than those from the other groups during any of the desiccation time periods.

An analysis of variance with interaction and a Duncan's test ($P = 0.05$) were used to determine whether any group showed significantly greater overall survival.

Length, width and height of each shell were measured to the nearest 0.05 mm with dial calipers. Length (L) was designated as the maximum distance from the anterior to the posterior shell margin, width (W), the maximum distance between the lateral shell margins, and height (H), the vertical distance from the ventral margin to the apex of the shell. In addition, the degree of steepness [$S = 2H/(L + W)$] and the degree of roundness ($R = W/L$) was determined for each shell. Both equations were used by Orton (1932) for *P. vulgata*.

The shell measurements were analyzed statistically to determine whether shells from the three habitats differed significantly in size and shape. A randomized block design analysis of variance and a Duncan's multiple range test ($P = 0.05$) were used.

TABLE II

Mean shell characteristics of limpets from vertical rock face, tide pool and subtidal habitats, $P = 0.05$

Habitat group	Mean length (mm)	Mean width (mm)	Mean height (mm)	Mean steepness	Mean roundness
Vertical	18.73	14.24	5.87	3.55	7.68
Tide Pool	14.58	11.03	4.45	3.46	7.60
Subtidal	17.82	14.42	5.37	3.33	8.07

RESULTS

Dessication experiments

As the length of the desiccation period increased, the number of limpets surviving decreased (Table I). Results of the statistical analysis showed that the mean per cent survival of the three habitat groups combined, at each time period, decreased significantly as desiccation time increased. Per cent survival under constantly submerged conditions (0 desiccation time) ranged from 99 to 100%. There was no survival in any of the three groups after 60 hours of desiccation. No specimens from the TPG and SG survived desiccation for 48 hours but a small per cent of the specimens from the VRFG were able to survive desiccation for 48 hours.

The per cent survival of the three groups did not differ significantly at 0, 48, and 60 hours of desiccation. At desiccation periods of 12, 24, and 36 hours, the VRFG had significantly greater per cent survival than the TPG. At 24 hours of desiccation, per cent survival of the VRFG was significantly greater than both TPG and SG. TPG and SG did not differ significantly at any time period.

The mean per cent survival of six time periods, determined for each of the habitat groups as part of the analysis of variance test, was 46.9% for the VRFG, 26.1% for the TPG, and 35.4% for the SG. The per cent survival of the VRFG was significantly greater than both the TPG and SG, and the mean per cent survival of the SG was significantly greater than the TPG. Fifty per cent survival occurred between 12 and 24 hours of desiccation for the TPG and SG, and between 24 and 36 hours for the VRFG.

Shell measurements

Mean length, width, height, steepness, and roundness are listed for each habitat group in Table II. The mean length and width of tide pool specimens were significantly smaller than the mean length and width of the VRFG and SG. There was no significant difference in mean length or mean width between the VRFG and SG. The mean height of tide pool specimens was significantly smaller than the mean height of the VRFG. There was no significant difference in mean height between the VRFG and SG. There were no significant differences in mean steepness or mean roundness between any of the three habitat groups.

Discussion

Several studies of the tolerance of intertidal gastropods to desiccation have shown that such tolerance was correlated with habitat level in the intertidal zone (Colgan, 1910; Gowanloch and Hayes, 1940; Davies, 1969). Individuals of a species inhabiting a high level tolerated desiccation better than low level individuals of that species. This study showed that in the limpet, *A. testudinalis,* tolerance of desiccation was not a characteristic only of specimens from the upper level of its vertical range. Specimens collected from upper level vertical rock faces and from lower level subtidal areas were more tolerant of desiccation than specimens collected from tide pools which were at an intermediate position.

This study showed that tolerance of desiccation in the three habitat groups was related to the size of the organisms. Mean shell sizes of limpets from the vertical rock faces and subtidal habitat were significantly greater than those of limpets from tide pools. The data regarding differences in shell size between limpets from the vertical rock face habitat and those from the tide pools are in agreement with data of Das and Seshappa (1948) who reported that small-sized *P. vulgata* could not tolerate exposure to desiccation, and only large-sized specimens of *P. vulgata* were found under conditions of exposure. In contrast, the results of my study do not support those of Segal (1956) and Shotwell (1950). Segal (1956) found that large and small *Acmaea limatula* were equally tolerant of desiccation. Shotwell (1950) observed that smaller specimens of *Acmaea pelta, A. mitra,* and *A. scutum* were more tolerant of desiccation than larger limpets.

Several studies have shown that a steep shell in limpets retards water loss and increases tolerance of desiccation (Orton, 1932; Moore, 1958; Ebling, Sloane, Kitching and Davies, 1962; Jobe, 1968). Davies (1969) found that individuals of *P. vulgata* inhabiting areas of exposure to desiccation had steeper shells, while those in damp areas had flat shells. He concluded that the relatively smaller circumference of the steeper shells resulted in a smaller surface area through which water was lost.

This study showed that in *A. testudinalis,* unlike *P. vulgata,* a steep shell with a small circumference was not characteristic of limpets which were most tolerant of desiccation. Statistical analyses of measurements of shell steepness and roundness showed no significant differences in shell shape in any of the three habitat groups.

Two tentative conclusions can be made regarding the origin of the size distribution pattern in *A. testudinalis.* Both require further investigation to substantiate. The first conclusion is based on an assumption that small limpets in tide pools are young, and larger limpets in the habitats above and below this level are older. The predominance of young limpets in the tide pools would then suggest that the larvae of *A. testudinalis* settle here and migrate outward as they mature. The larvae may settle on rock surfaces which are exposed at low tide, or subtidally, but do not survive in these habitats. Small limpets are herein shown to be least tolerant of desiccation. They may also be more vulnerable to predators such as sea stars which occur subtidally. Blackmore (1969) stated that population pressure caused the outward migration of maturing *P. vulgata* from larval settling areas low in the intertidal zone. Population pressure might also cause the outward migration of *A. testudinalis* from tide pools.

A second explanation of the size distribution pattern in *A. testudinalis* is proposed on the basis of Sutherland's (1970) study of the population dynamics of *A. scabra*. He concluded that size distribution in this species was determined by population density. Low recruitment of limpets in the upper zone, resulting in low population density, permitted the limpets to grow faster and larger at this level. In the lower zone, high recruitment and resultant high population density inhibited growth. A study of the population dynamics of *A. testudinalis* may reveal a similar relationship between size distribution and population density. Perhaps larger numbers of larvae settle in tide pools than above and below this level. Resulting lower population densities in the vertical rock face and subtidal habitats may promote more rapid growth, hence populations of larger limpets at these levels.

The VRFG and SG showed some differences in ability to survive desiccation. Per cent survival of the VRFG was greater than that of the SG at desiccation periods of 12 through 48 hours. (Table I). This difference was consistent although it was significant only at 24 hours. Fifty per cent survival in the VRFG occurred between 24 and 36 hours, while that for the SG occurred earlier, between 12 and 24 hours. Mean per cent survival for the VRFG was significantly greater than the SG. These differences in survival could not be correlated with any differences in size or shape. The factor responsible for the greater degree of survival in the VRFG may have been physiological. Davies (1969) reported that specimens of *P. vulgata* inhabiting the high intertidal zone were better able to limit the rate of body water lost under desiccated conditions than specimens from the low intertidal zone.

The limpets collected from vertical rock faces were found between 0.5 and 1.5 m above mean low water, the upper limit of the intertidal range of this species, where they would be exposed to a maximum of six to eight hours of desiccation on a low tide. Most VRF specimens tested in these experiments were able to survive at least 12 hours. The limpets collected subtidally and from tide pools were never subjected to desiccation in these habitats, yet a high percentage were able to survive 12 hours under experimental conditions.

Thus other factors probably interact with desiccation to limit the distribution of *A. testudinalis*. Other studies have indicated that desiccation alone does not determine the upper limits of an intertidal species (Stephenson, 1942; Test, 1945; Davies, 1969). Temperature, wind and degree of insolation may lower an organism's tolerance of desiccation (Stephenson, 1942). Grazing gastropods, with a strict food preference, may be limited to a lower habitat by their algal food supply which grows at a lower level (Test, 1945). Davies (1969) found that the upper limit of the range of *P. vulgata* was below that which would cause lethal water loss. He suggested that there was a balance between amount of body water lost on exposure and the time required to recover this water when the limpet was submerged during the next high tide. Further study is necessary to determine the factors which may interact with desiccation to limit the vertical distribution of *A. testudinalis.*

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SUMMARY

1. This study examined tolerance of desiccation in the limpet, *A. testudinalis,* collected from two intertidal habitats, vertical rock faces and tide pools, and one subtidal area on Schoodic Peninsula, Hancock County, Maine.

2. The results of two laboratory desiccation experiments showed that subtidal limpets and those from vertical rock faces were more tolerant of desiccation than those from tide pools.

3. Greater tolerance of desiccation was related to the size of the organisms. Mean shell sizes of subtidal limpets and those from vertical rock faces were significantly larger than those of limpets from tide pools.

4. Shell shapes of limpets from the three habitat groups did not differ significantly, which showed that differences in tolerance of desiccation were not related to differences in shell shape.

5. Limpets from vertical rock faces showed a slightly greater tolerance of desiccation than those from the subtidal area.

6. The size distribution of limpets on Schoodic Peninsula suggests either that larvae of this species may settle in tide pools and migrate outward to exposed intertidal and subtidal habitats as they mature, or that population density in each habitat determines the rate and limits of growth of the resident limpets.

7. This species tolerated more desiccation than it would be required to withstand in its natural habitat. Desiccation must act with other factors in determining the upper limit of the distribution of this species.

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PHOTIC SIGNALING IN THE FIREFLY *PHOTINUS GREENI*

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It has been known for many years that most lampyrid fireflies use their light emission to bring the sexes together. Often the attraction is achieved by a characteristic signal system or code which enables male and female of a particular species to recognize each other even in the presence of other species. In many members of the genus *Photinus* the courtship exchange involves a flying male, flashing in a fixed rhythm, and a perched female who does not flash spontaneously but only in response to a proper signal. In some species the specificity of the code is known to depend on the female always responding at a fixed interval after seeing an acceptable signal and upon the male recognizing that interval. Within considerable ranges, color, intensity and duration of flash are not critical to the signals of either male or female, and flashes of artificial light can be substituted for either firefly in the dialogue.

Firefly codes are interesting physiologically because they afford clues to the neural control of flashing. It is known that the rhythmic flashing of the male is controlled by a pacemaker in the brain and that the timing of both the male's cycle and the response flash of the female can be influenced by light signals received through the eyes. These responses require visual processing, central nervous processing and conduction of the excitation through the main nerve trunk and peripheral nerves into the light organ and along a pathway of several steps in the luminous tissue. The minimum time occupied by this composite excitation process, the physiological "latency," as measured after stimulation of the eye by light or electricity in a number of species, is in the 150–250 msec range at 25° (Case and Buck, 1963; Buonamici and Magni, 1967; Magni, 1967; Case and Trinkle, 1968; Papi, 1969). Almost always, however, the actual behavior in nature involves far longer delays. For example, the response delay of the female of *P. pyralis* averages about 2 seconds, or about 10 times the minimum eye-to-lantern latency (Buck, 1937; Lloyd, 1966b). It is clear, therefore, that actual firefly codes are constructed by incorporating different but fixed amounts of central nervous delay into the overall perception-response circuit.

In *P. pyralis,* where both male and female emit only single flashes, there is no ambiguity about time relations within the signal code. In the *P. consanguineus*

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complex, however, where the male emits flashes in pairs (McDermott, 1910, 1911, 1912, 1914, 1917; Lloyd, 1966a, 1966b, 1969) some interesting questions arise. It is clear that both flashes in the male's species "phrase" (Lloyd) are necessary for communication, since the female will only very rarely respond to a single flash, but it is not known what functions the two flashes perform. One could imagine, for example, that the female times her response either from the first of the male's flashes (the second serving only a confirmatory or reinforcing role) or from the second (the first perhaps serving to prepare or "prime" her response system).

Apart from physiological considerations, analysis of firefly codes bears importantly on the species concept and the evolution of communicative behavior. Barber (1951), McDermott and Buck (1959) and Lloyd (1966b, 1971) have adduced considerable evidence for the behavioral separation of forms that are nearly or entirely indistinguishable by conventional morphological criteria but are presumably isolated reproductively by the specific codes. Since this work has been based largely on visual observation it is clearly desirable to document the codes as quantitatively as possible and explore the limits of variation within the signal systems—that is, the ranges of timing, duration and shaping of the male's signal in relation to its acceptability to the female, and the extent to which the female's latency can vary without breaking contact with the male. The *consanguineus* group is ideal for such study both because its code is relatively simple and because the species complex appears to consist of only a few members. The group is presently divided into three species, *P. macdermotti* (Lloyd, 1966a), *P. greeni* (Lloyd, 1969) and *P. consanguineus* s.s. (Lloyd, 1966a, 1966b, 1969) on the basis mainly of apparent differences in timing within the phrases of the males and in the response intervals of the females. However, all three species range widely over the Eastern United States and show enough local variation in behavior and morphology to confuse their interrelations.

A prime reason for attacking the above-mentioned problems by a field study prior to laboratory investigation is the well-known tendency for fireflies, particularly males, to flash abnormally in captivity. In addition, the consistent differences sometimes found between individuals argue for the sampling of a considerable population.

MATERIAL AND METHODS

The species studied is one that is active in Woods Hole, Mass. in late July and the first three weeks of August. Though designated "*consanguineus*" in a preliminary report (Buck and Buck, 1965) it more closely resembles the later named *P. greeni* in behavior and is provisionally so identified though differing somewhat in morphology from the type of that species, described from Florida. The Woods Hole form begins its evening activity at about 8 P.M. (EDST) when the surround is still quite light. The flight of the male is mostly over within an hour, though responsive females can be found for an additional hour.

The experimental work concentrated on exploring the modifiability of the male's signal, substituting the light of an ordinary pushbutton flashlight or electric torch for the male in dialogues with females in the field. However, enough males were attracted, using torch in place of female, to confirm the expected importance in courtship of the interval between the male's second flash and the female's response flash. As is usual in such simulated courting, the beam of the torch was not pointed directly at the firefly. Rather the intensity of the signal was adjusted crudely to decreasing distance by shielding it more and more against ground vegetation. Even so, both the intensity of the light reaching the firefly's eye and the apparent area of the (reflected) source must have varied enormously.

In these exchanges the various flashes were recorded by means of a portable single-channel photometer, developed by Dr. Frank Hanson, which makes use of an RCA 1P21 photomultiplier and Cambridge Transcribe chart recorder. Since the flashes of the fireflies and the incandescent torch lamp had very similar rise times (*ca.* 50 msec), latencies and interflash intervals in the chart records were measured peak to peak. Because of variable orientations and distances of fireflies and torch vis a vis the photometer, apparent intensities of the recorded flashes are not comparable. Since flash frequency and response times are also highly dependent on temperature, absolute values have little significance *per se*.

Statistical parameters used are the standard error (indicated by "±") and the coefficient of variation "V."

RESULTS

1. *Courtship dialogue*

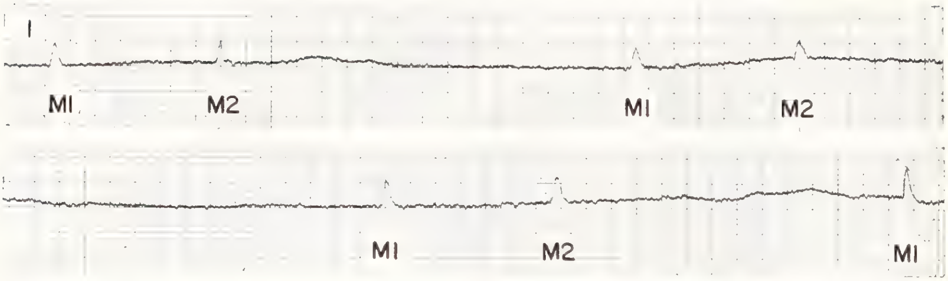
The female of *P. greeni*, though fully winged, does not ordinarily fly but perches on vegetation near the ground. The male flies only a meter or so above the ground, repeating a two-flash phrase about every 5 seconds (at 27°) (Fig. 1). When a male flashes within range of a female (up to 6 or 7 m depending on environmental illumination and terrain) the female replies after his second flash (Fig. 2). The male then advances toward her in a succession of such exchanges and eventually alights near her and completes the journey on foot. The interphrase interval of the male is not critical, since females will respond to an actively searching male (or torch) that leaves only three seconds or so between phrases or will remain responsive over many minutes during which no stimulus is received.

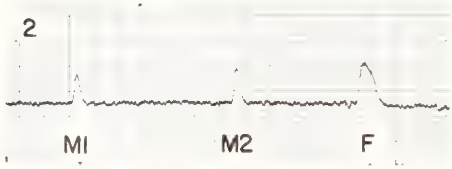
When the male of *P. greeni* breaks rhythm, as for example when abandoning a nonproductive dialogue, the interruption comes after the second flash of the phrase, not between flashes of the pair. This supports the expectations that the flashes constituting the species-specific pattern are delivered as a sequential unit, whereas the phrase-repetition rate, though usually quite regular, can be interrupted or varied to a much greater extent.

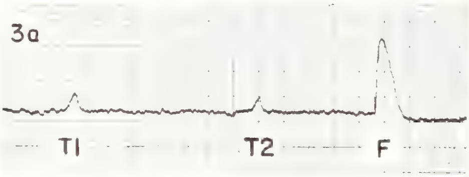
The female's flash is surprisingly bright considering that her lantern is only about a quarter of the area of the male's. Her flash is more than twice as long as that of the male (*ca.* 220 msec—Figs. 2–7). As in many other photinids the female flexes and twists her abdomen when delivering her response flash so that the surface of the lantern faces toward the male.

2. *Flash periodicity in male*

The male of *P. greeni* emits bright sharp flashes of yellowish light lasting about 100 msec. The interval between the two flashes of the phrase averaged 1.30 ± 0.01 sec in a male during 10 exchanges with a female, 1.35 ± 0.007 sec in







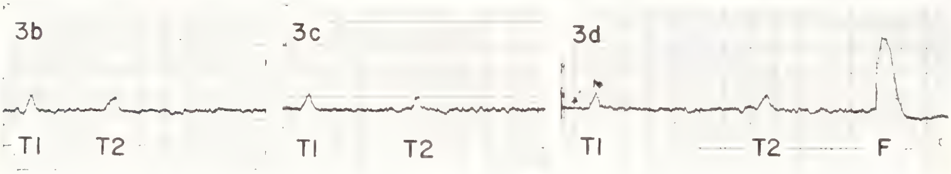


FIGURE 1. Normal spontaneous flashing of male of *P. greeni* in flight while investigating torch answers. Continuous record of 3½ successive phrases; time scale in this and all photometer records = 200 msec between heavy vertical lines.

FIGURE 2. One signal-answer episode between male on foot (M) and female (F).

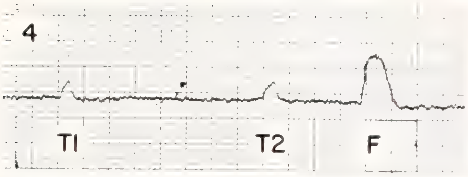
FIGURE 3. Four successive episodes in torch signaling with female; about 3 second gaps between episodes. Note that female fails to respond when the interval between the two flashes of the "male" is too short (Fig. 3b, c). (The female of Figs. 2 and 3 is the same as female No. 4 in Fig. 8).

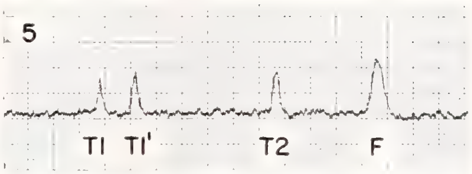
the same male during 19 periods of unengaged flashing in flight, and 1.26 ± 0.02 sec in a second male during 12 cycles in flight during which attempts were being made to lure him to the torch (all at 27°). The inconsequential differences indicate that the male's phrase-making is independent of visual input from the female.

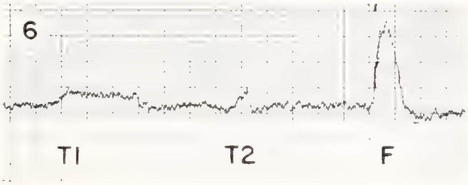
The period of repetition of the phrase was 4.85 ± 0.013 sec in a total of 22 periods of two males in searching flight at 27°. The question of whether the male's pacemaking rhythm is different during actual dialogue was not studied but no difference was noticed. However, when the male has alighted and is proceeding toward the female on foot, which happens during the final stages of courtship, the intervals between successive phrases are apt to be markedly irregular.

3. *Female's response delay*

At 27° two females in dialogue with males responded, respectively, after mean delays of 0.92 sec (2 cycles) and 0.94 ± 0.02 msec (range 0.86 to 1.1; 10 cycles), measured from the second of the male's paired flashes. These females and several







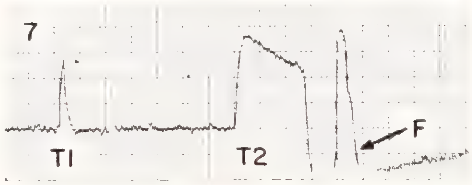


FIGURE 4. Response of female to paired flashes from torch.

FIGURE 5. Response of female to torch signals in which an extra flash (T'_1) is intercalated.

FIGURE 6. Response of female to torch signals in which the duration of T_1 is greatly increased.

FIGURE 7. Response of female in which the duration of T_2 is greatly increased. The dip in the photometer trace is an electrical artifact. The trace has been strengthened for reproduction. (The female of Figs. 4–7 is as female number 5 in Fig. 8).

others were tested with a total of 220 paired torch flashes. The delays recorded in the 127 responses are summarized in Table 1. This table shows that the responses of females to paired torch flashes involved the same order of delay as in dialogues with real males even though the intervals between the two torch flashes were in many instances well outside the range of intra-phrase intervals of actual males (extreme range among two males, 1.16 to 1.42 secs; among effective torch pairs 0.92 to 2.20 secs).

4. *Intraphrase interval*

The frequency distributions of the 127 responses and 93 non-responses to the 220 torch signals to the 6 females (Table 1) are plotted in Fig. 8. Granted that

TABLE I

Time relations during simulated courtship signaling. (T_1 = first flash of simulated male phrase; T_2 = second flash; F = response of female)

Date	T°	No. resp.	$T_1 - T_2$		$T_2 - F$		$T_1 - F$	
			$\bar{M}$ S.E.	V	M S.E.	V	$\bar{M}$ S.E.	V
VIII/14	(22°)	20	1.54 ± 0.03	0.09	0.96 ± 0.02	0.08	2.50 ± 0.03	0.06
VIII/15	(27°)	21	1.58 ± 0.06	0.16	0.91 ± 0.02	0.08	2.47 ± 0.06	0.11
VIII/17	(27°)	29	1.31 ± 0.02	0.08	0.94 ± 0.01	0.06	2.25 ± 0.02	0.06
VIII/18	(27°)	18	1.37 ± 0.04	0.12	0.84 ± 0.02	0.08	2.21 ± 0.04	0.07
VIII/18	(27°)	30	1.38 ± 0.04	0.15	0.80 ± 0.01	0.08	2.19 ± 0.04	0.10
VIII/18	(27°)	9	1.21 ± 0.08	0.20	0.93 ± 0.04	0.11	2.14 ± 0.06	0.09

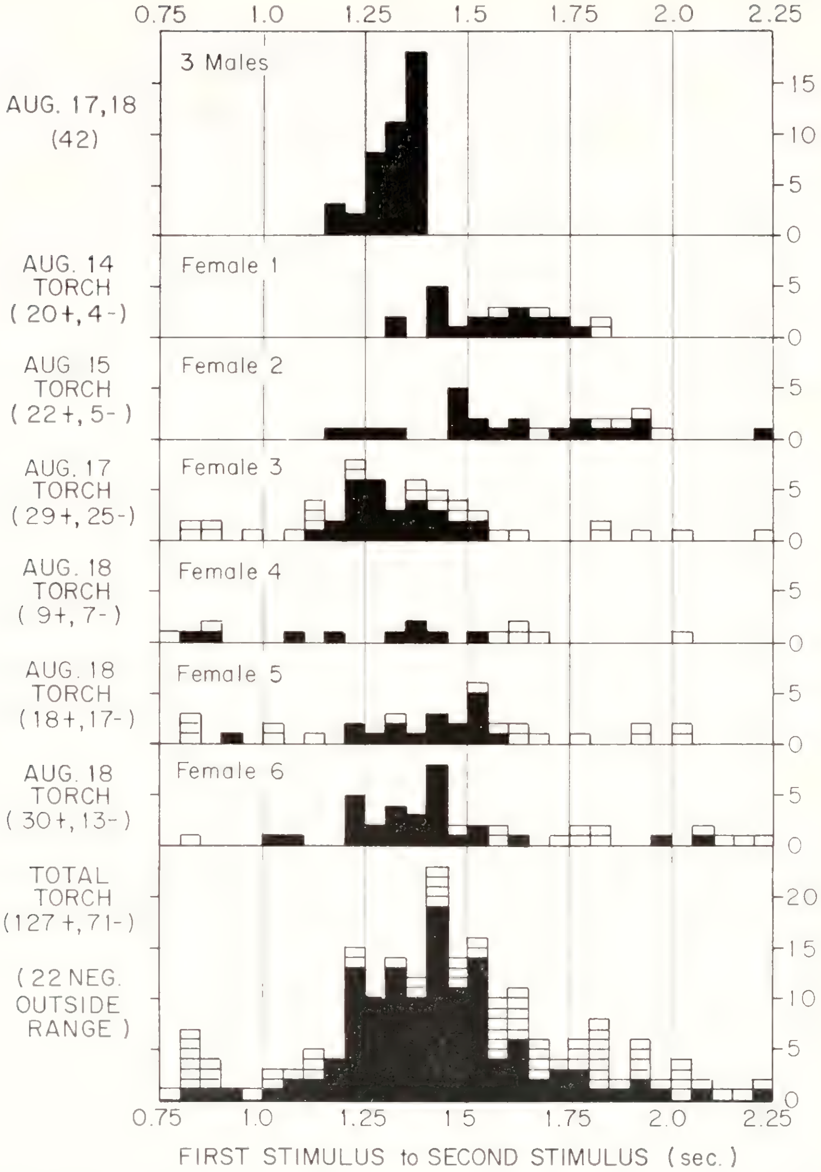


FIGURE 8. Frequency distributions of natural phrase durations in three male fireflies (top line) and experimental torch phrases used in signaling to six different female fireflies (next six lines: last line shows total for torch signals). Black units indicate responses, white units are failures to respond.

failure to respond is much less significant than response (because a female presumably may fail to react by reason of the artificial flashes being too bright, too dim or too large in area, or for unknown reasons, as well as because of unacceptable timing) and that response failure by the female is not rare during natural signaling in the field, it is apparent that the range of acceptable intra-phrase flash intervals is comparatively narrow. In five of the females the range within which a reasonable percentage of responses occurred was about 750 msec and in the sixth about 500 msec. Even so the range appears to be at least twice the actual spread of phrase intervals in male fireflies (top line, Fig. 8).

5. *Phrase format*

To explore the informational significance of the two flashes of the male's signal phrase each was modified separately in number and in duration in a number of torch simulation tests. T_2 proved much less labile than T_1, but disregarding failures of response these tests showed that either flash can be repeated at least once within a period of 250 to 500 msec or lengthened up to 820 msec without vitiating its role as an essential part of an effective stimulus to the female. Examples of such effects are given in Figures 5, 6 and 7.

As an additional probe of the criteria of acceptable stimulus the two flashes of the phrase were delivered by torch from opposite sides of the female. When the first of the stimulus pair was thus received mainly by one eye and the second by the other eye the female nevertheless responded normally in each of many trials.

6. *Male-male interactions*

On several occasions when a female was being stimulated repetitively by torch signals, flying males approached the scene, fell into step with the torch and entered into dialogue with the female. Except for the remote possibility that the flashing rhythm of these males just happened to be in the proper phase relation to the female's response to the torch at the time the male flew into range, such behavior indicates that the male recognizes not only the female's response delay interval but its relation to the preceding stimulus flashes.

7. *Starting point for female's response latency*

If the range of acceptable intra-phrase intervals could be made sufficiently large in proportion to the range of response latencies of the female it might be possible to deduce whether the female times her response from the first or from the second flash of the stimulus phrase. The argument would be that if the latency were timed from T_1, the T_1-T_2 interval should be inversely related to T_2-F whereas if timed from T_2 the two values should be independent. It would also be expected that T_1-F might be more variable than T_2-F if the latency is timed from T_2.

Figure 9 presents a scatter diagram for the 127 pairs of effective torch phrase intervals and the corresponding latencies for the six females (Table 1 data). The distribution is consistent with independence of T_1-T_2 and T_2-F, although the variability of T_2-F is great enough to weaken the conclusion. If the T_1-T_2 values are divided into two populations on the basis of being shorter than or longer than 1.4 seconds ($\overline{M} = 1.24 \pm 0.015$ for 60 phrases; $\overline{M} = 1.56 \pm 0.021$ for 67 phrases) the

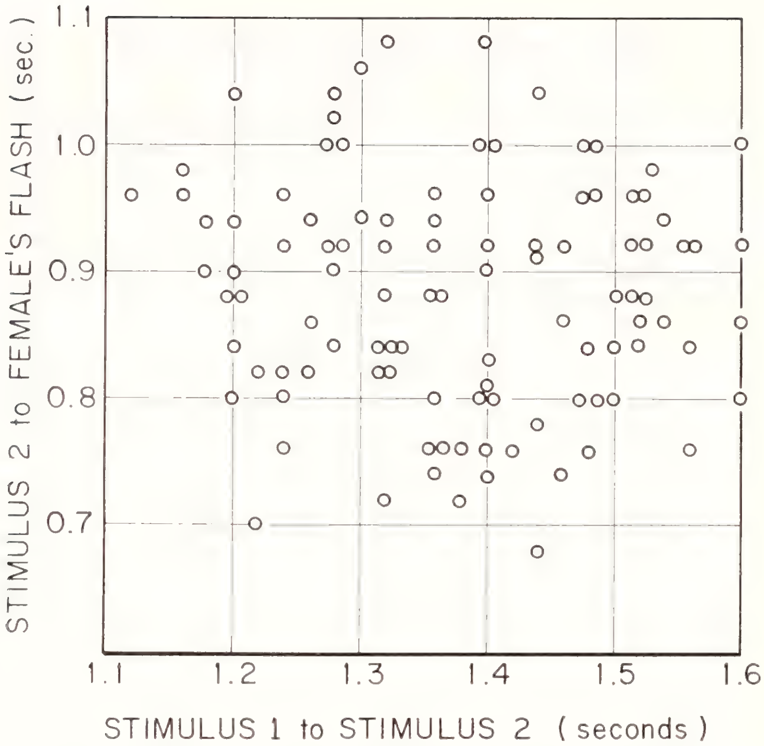


FIGURE 9. Relationship between 127 paired torch phrases (abscissa) and corresponding latencies of responses of females (ordinate); total for 6 dialogues.

corresponding T_2-F values are 0.89 ± 0.01 and 0.89 ± 0.01, again indicating independence of T_1-T_2 and T_2-F. However, the fact that the coefficients of variation of T_1-F and T_2-F are not systematically different (Table 1) somewhat militates against distinguishing a starting point for the latency in this way.

DISCUSSION

Probably the most significant conclusion to be drawn from experimental signaling to females of *P. greeni* in the field is that response can be elicited over wide ranges of signal intensity, duration, source area, format and orientation providing only that the two flashes of the stimulus phrase are separated by the proper interval (roughly 1.1 to 1.7 secs at 27°). This fact supports previous evidence of the primacy of time relations in firefly signaling systems but extends it to systems a step up in complexity from that of species like *P. pyralis,* where the proper stimulus to the female is a single flash. In *P. greeni* the female must be able to recognize as an informational unit a pair of flashes separated by a particular range of intervals. In this connection it is well to keep in mind that the female's response is a cue for the male to orient, not to flash, whereas the male's phrase is a cue to the female both to orient (her abdomen) and to flash.

Though these rough experiments in no sense define the female's receptor requirements, the pulse-duplication tests show that she becomes in effect refractory for several hundred milliseconds after stimulation. Thus, when the first flash of the male's phrase is doubled, the second element (T'_1, Fig. 5) does not supercede the initial flash (T_1) as the starting point of a response cycle nor does it take over the position of T_2 as time zero for the latency of the female's eventual response. Whether this refractoriness resides in the response mechanism or in the receptor part of the excitation system must remain moot at least until the flicker fusion frequency of the female's eye can be measured. The fact that the time of *ending* of a flash does not have any effect on the timing of the female's response even when the flash is long enough to have exceeded the ordinary limits of neural refractoriness (T_1, Fig. 6; T_2, Fig. 7), suggests that only an *increase* in light intensity can qualify as a stimulus. Such a conclusion would be consistent with work on signaling in *Luciola lusitanica* in which Papi (1969) demonstrated the importance of the rate of rise of light intensity during the stimulus flash.

The experiments in which the two flashes of the phrase were delivered alternately from opposite sides of the female argue that the mechanism that is successively activated by the two events is, as expected, central, not peripheral and that the dialogue does not require a special relative orientation of male and female.

The fact that the female's latency is unchanged with changing stimulus pulse duration again argues that the effective part of the flash is the initial rise in light intensity—*i.e.,* that the female's response is an "on" response.

The fact that the female accepts a considerably wider range of intraphrase intervals than is normally provided by actual males (Fig. 8) is in line with Lloyd's suggestion (personal communication) that a workable code, beside being exclusive of those of other species of firefly active at the same time, must allow for the likelihood that a male in flight may have a considerably different body temperature from that of a female perched near the ground.

Durations of interflash intervals in different species of fireflies in different localities and of fireflies studied by different investigators should be compared with caution, particularly when measured at nominally different temperatures. However, it appears that the interflash interval in the male's stimulus phrase in the Cape Cod *P. greeni* agrees with that of the Florida form in being distinct from Lloyd's figures for the Florida strains of *P. consanguineus* (*ca.* 0.5 sec at 26°) and *P. macdermotti* (2 secs at 23°) and the Maryland strain of *macdermotti* (1.8 sec at 24°). Our mean value of about 1.3 sec at 27° seems disturbingly different from Lloyd's Florida value of 1.2 sec at about 19°, but our response latency for the female (*ca.* 0.9 sec at 27°) is quite close to Lloyd's figure of 1 sec at 24°. Though the temperature is not given, Carlson's (1969) data for laboratory tests indicate that his Long Island species could also be *P. greeni.* Thus the validity of *P. greeni* as a separate species seems to be supported, as is the mutual independence of *greeni, macermotti* and *consanguineus: i.e.,* the males' phrases are probably sufficiently different to be distinguishable by the respective females.

The ability of a male photinid to recognize that a courtship exchange between a female and another male (or torch) is underway and to shift his flashing rhythm so as to coincide with that of the engaged male, was first noticed among coteries of *P. pyralis* males in natural courtship (Buck, 1935) and confirmed in torch exchanges in the same species (Buck and Buck, 1968). Lloyd (1971) has illustrated an interesting instance of similar ability in *P. greeni:* A male, after having seen an artificial two pulse pattern and the female's response, was able to supply the second flash of a stimulus phrase in which the first flash was artificial light. Male-male synchronization has recently been analyzed in a New Guinea *Pteroptyx* (Hanson, Case, Buck and Buck, 1971) and appears to be quite different from that of the courtship signals. In any case the coexistence of two such well-developed communication systems in one species of *Photinus* seems remarkable.

The question of the takeoff point for the female's response latency is of sufficient interest neurologically that it would be desirable to have more conclusive statistical evidence of the independence of T_1-T_2 and T_2-F. More T_1-T_2 intervals at the extremes of the acceptable response range are needed, a desideratum requiring more precise and reproducible signal control than possible with a manual pushbutton. However, an additional indication that the female times her response from T_2 is provided by a few instances in which the female responded to the first of the two flashes of the phrase. In such instances the latency measured was in the usual T_2-F range.

We thank Drs. Frank Hanson, James Lloyd, Albert Carlson and Margaret Peterson for various favors.

SUMMARY

1. In duplicating the courtship signals of *Photinus greeni* it was found possible to substitute flashes of artificial light for either the male or the female.

2. In confirmation of previous work, the courtship signals of *P. greeni* were found to depend on the female's recognition of the male's stimulus signal of two flashes and the male's recognition of the female's fixed response interval after his second flash. At 27° C the acceptance limits of the male's phrase were from about 1.1 to 1.7 seconds and the limits of the female's response latency were from about 0.8 to 1.1 seconds.

3. Within wide limits the delivery rate of the male's two-flash signal ("phrase"), and the intensities, durations and source areas of the flashes of male and female, are immaterial to the success of the signaling.

4. The two flashes of the male's stimulus phrase can be made multiple, or increased several-fold in duration, without preventing the female from responding, as long as the proper time relation between the starts of the first flash and one subsequent one is preserved. From this it is concluded that it is the rise in light intensity that is perceived by the female and that she is refractory to new visual input for several hundred milliseconds after being stimulated.

5. Instances in which males fell into step with torch flashes being used to stimulate females serially are interpreted as male-male communication.

6. It is concluded tentatively that the female times her response latency from the second flash of the male's stimulus phrase.

7. The behavioristic data for the Cape Cod *P. greeni* are consistent with its belonging to the same species as the Florida strain and with being distinct from *P. consanguineus* and *P. macdermotti.*

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DEVELOPMENT AND JUVENILE GROWTH OF THE SEA ANEMONE, *TEALIA CRASSICORNIS*

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The development of *Tealia* (= *Urticina*) *crassicornis* (Müller, 1776) has been studied by Appellöf (1900) who reported that in Europe this species releases its gametes freely into the sea and the larval development is independent of the adult. Hand (1955) reported this species as being a brooder. In this paper we present information showing that *T. crassicornis* in the Friday Harbor area has a mode of development similar to that described by Appellöf (1900). We have followed the embryonic and larval development by histological techniques and although our results conform largely to those of Appellöf, we differ from him in several interpretations of major importance. We have successfully reared the larvae to metamorphosis; the young anemones in our laboratory are now 18 months old. Our findings on the induction of larval settlement by worm tubes and on the effect of feeding on juvenile growth should also prove to be significant in anthozoan biology.

MATERIALS AND METHODS

Tealia crassicornis, collected from the San Juan Island area and kept in the sea water tanks at the Friday Harbor Laboratories, spawned several times during the springs of 1970 and 1971. The eggs and sperm were collected as they were released. The eggs were put into covered culture dishes containing 500 ml of sea water, and a small amount of sperm suspension introduced. After approximately one half hour the eggs were rinsed free of sperm. The water was subsequently changed periodically. Development was allowed to continue in these dishes at approximately 12° C. After being kept four months in the culture dishes some of the juveniles were removed and placed on glass microscope slides. Once they attached, the slides were placed in vertical holders in a "Plankton-Kreisel" (Greve, 1968) as modified by Roosen-Runge (1970). The young anemones in the dishes and the "Kreisel" were fed *Artemia* during the first five months. Starting with the sixth month young anemones in the "Kreisel" were fed pieces of mussel or shrimp meat, while those in the dishes were starved.

For histological preparations, individuals at various stages of development were fixed in Bouin's fluid and embedded in paraffin. Serial sections were cut at 5 μ thickness and stained with Heidenhain's hematoxylin and Orange G.

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Spawning

On four occasions in May and June, 1970, and once in April, 1971, animals were seen to spawn in the laboratory. At least one of each sex spawned every time, and thus all eggs which were allowed to float in the tank were fertilized. In all cases, animals spawned in the morning between 8 and 11 o'clock.

When spawning, the tentacles and the column of the animal were fully extended. Strings of mucus containing gametes were expelled gradually from the mouth, in the manner of undigested food particles being ejected. Eggs, immediately after being expelled from the mouth, attached to each other to form small clusters. They tended to sink, but fell apart before reaching the bottom of the tank, and began to float on the surface of the water. Sperm, on the other hand, became suspended soon after being discharged from the mouth.

While spawning, animals remain still and appear to be somewhat insensitive to mechanical disturbance. For example, probing of a non-spawning individual always causes the retraction of tentacles, but when eggs are collected among the tentacles by using a basting syringe the animal rarely reacts.

Gametes

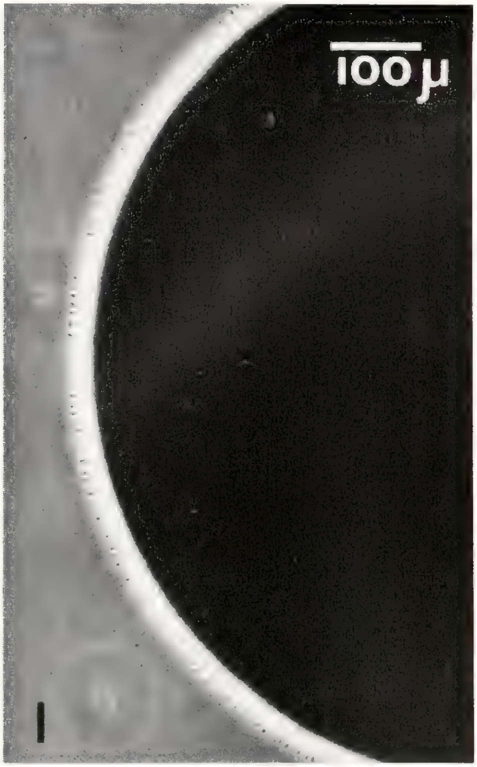
When expelled, the egg is spherical, yellow or tan in color, and measures from 500 to 700 μ in diameter. It flattens into a pancake shape when placed on a slide and the egg membrane can be easily ruptured in handling, allowing a stream of cytoplasm to escape. The surface of the egg bears numerous spines (Fig. 1), a characteristic of many anthozoan eggs. The spines in this species are relatively blunt, and are 25 μ in length. Meiotic division must have been completed before spawning, as neither germinal vesicle nor polar bodies were observed.

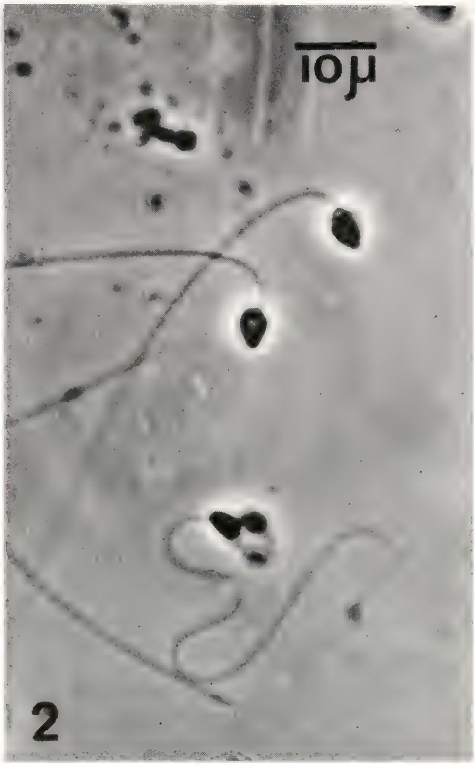
Histological preparation shows that there is a thin (5 μ) cortical layer of fine and dense cytoplasm without yolk platelets. Beneath this layer, there are a number of basophilic, presumably cortical granules, otherwise the oöplasm consists of yolk platelets of two kinds: lipid yolk which become vacuoles (5 μ diameter) after fixation in Bouin's Solution and paraffin sectioning, and small protein yolk granules, 2.5 μ in diameter (Fig. 15).

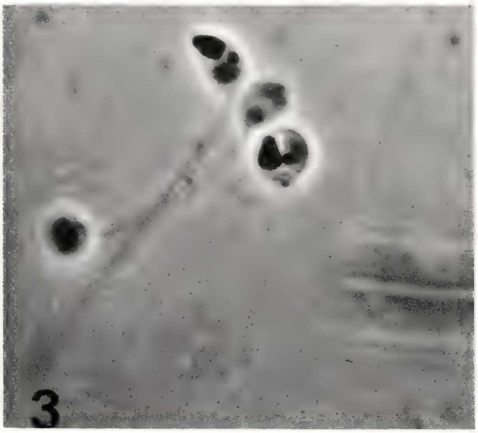
The sperm of this species are unique, in that not all of them are mature when discharged. In many of them there is still considerable excess cytoplasm housing the nucleus and the middle piece which is attached to a dark body of unknown nature. These two components are seen moving about in the cytoplasm; they may either be separated from each other (Fig. 4) or clumped (Fig. 3). The tail in the meantime beats vigorously. The sperm head is more or less oval in shape, measuring 2 × 1.5 μ in size, and the tail is 60 μ long (Fig. 2). It is not certain whether or not the immature sperm are functional, but the discharge of these sperm is consistent in all the cases we have examined.

Cleavage

We were puzzled when first examining the eggs superficially in culture dishes, as we could not see any sign of cleavage furrows, even 24 hours after insemination. We discovered later that the cleavage is meroblastic and the first few cleavages







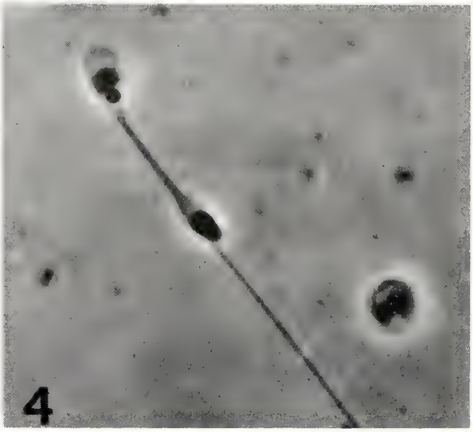
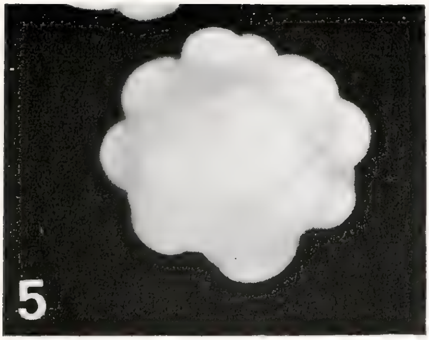


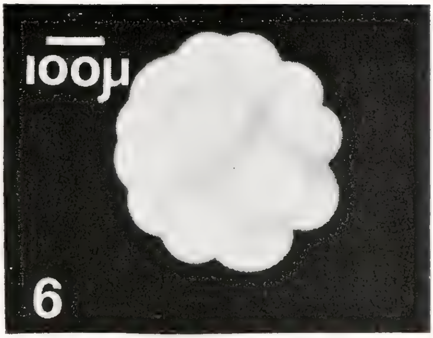
FIGURE 1. Portion of a freshly spawned egg, showing the spiny surface, phase contrast.

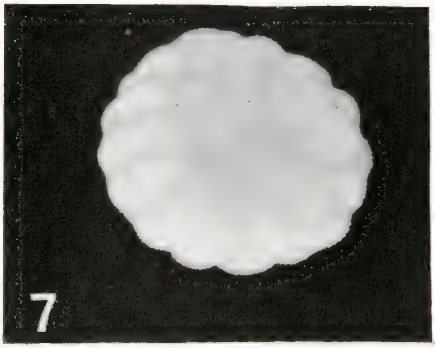
FIGURE 2. Mature spermatozoa, phase contrast.

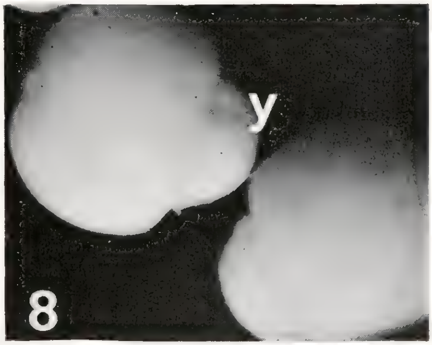
FIGURE 3. Immature spermatozoa, showing the head and the middle piece are clumped together in an excessive amount of cytoplasm, phase contrast.

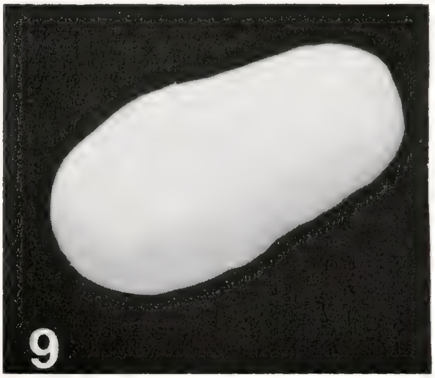
FIGURE 4. Immature spermatozoa, showing the head and the middle piece are separated, phase contrast.











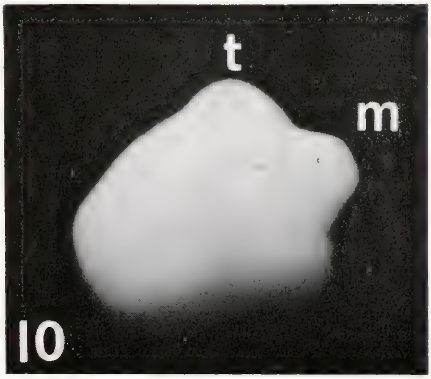


FIGURE 5. Vegetal view of early cleavage stage, showing the superficial nature of cleaving furrows.

FIGURE 6. Animal view of early cleavage stage.

FIGURE 7. Animal view of late cleavage stage.

FIGURE 8. Gastrula, showing a bulged "yolk plug" (y) at vegetal end.

FIGURE 9. Planula, apical end at the left hand side.

FIGURE 10. Metamorphosing planula, showing the protruded mouth (m) and the developing tentacle bud (t).

are confined to the animal pole which is heavier and floats toward the bottom of the dish. Further examinations of living embryos and subsequent histological studies confirmed that the nucleus divided several times before the cell membrane began to form, first at the animal pole, then at the vegetal pole, resulting in incomplete small blastomeres at the animal half (Figs. 6 and 7) and large blastomeres at the vegetal half (Fig. 5). The result of such superficial cleavage is a blastula with a single layer of poorly defined cells on the surface and a mass of uncleaved yolk in the center (Figs. 16 and 17). No blastocoel ever existed. The outer layer of the superficial cells consists mostly of the fine and basophilic cytoplasm of the cortex (Figs. 16, 17). After histological preparation, the surface spines are clumped together and reduced to 11 μ in length (Figs. 15, 17).

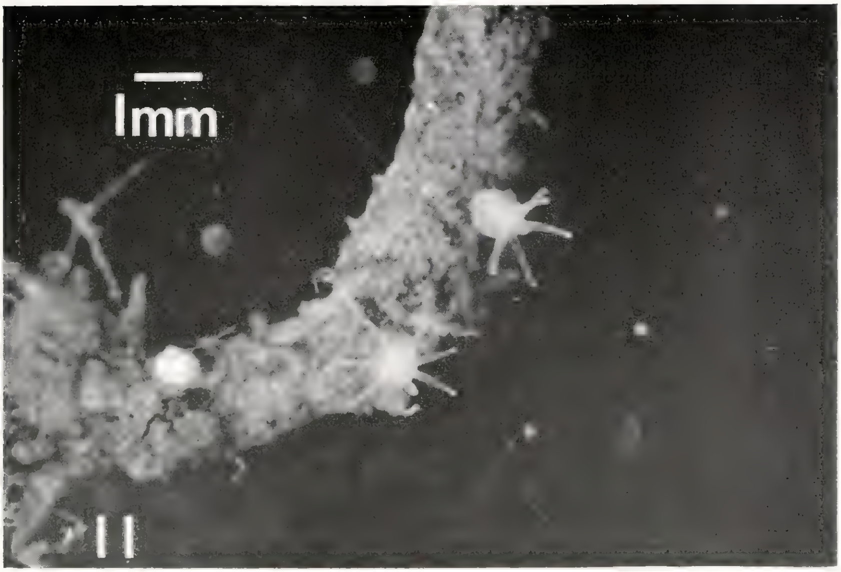
Endoderm formation (gastrulation)

In most of the known cases, gastrulation in anthozoa is accomplished by invagination; hence, the formation of endoderm is by emboly (Mergner, 1971) as far as we can ascertain. In the present species (as in *Halcampa* (Nyholm, 1949) and many of the Hydrozoa) the endoderm appears to arise by multipolar ingression. At gastrula stage, some cells must have migrated into the interior to establish two germ layers: the ectoderm and endoderm. Because of the presence of the yolk mass at this stage, the endoderm is difficult to define in histological preparations. Furthermore, these cells do not form a continuous layer until the planula stage and before that the endoderm cells are scattered with rather ill-defined boundaries.

The gastrulation is also marked by the formation of a structure, similar in appearance to the yolk plug of an amphibian gastrula, at the vegetal pole (Fig. 8). The formation of this "yolk plug" is apparently by the process of epiboly. At this time the embryo begins to elongate along the animal-vegetal axis to become ovoid in outline. The "yolk plug" was large and protruded at the beginning, but soon decreased in size owing to the rapid proliferation of the ectoderm cells of the animal half. In addition, yolky material is being absorbed, leaving the blastopore open as the mouth (Fig. 20). At this stage, the surface spines have disappeared, cilia have developed and the embryo becomes free-swimming. This is then a young planula larva.

Planula

The planula is cone-shaped (Fig. 9), 530 × 750 μ in size, swims with the apical end forward and spends most of the time swimming close to the bottom of the dish. There is no apical tuft as found in many other anthozoan planulae but an invagination (Fig. 18) at the apical end is consistently found in serial sections of the late gastrulae. The apical end of the planula stage no longer shows the invagination, but a group of cells in this area have clearer cytoplasm and lack the basophilic apices characteristic of the cells of the general ectoderm. Histological sections show that the ectoderm is a layer of simple columnar epithelium, and the cells show a considerable degree of differentiation: nematocysts, secretory cells, and spindle-shaped sensory cells (Fig. 19). The endoderm is a layer of simple cuboidal epithelium and is still very rich in yolk granules. Contractile elements have appeared in the endoderm of the body wall and mesenteries, hence the ability of the larvae to change shape. The central mass of yolk, left from earlier stages, is being absorbed, thus the larva becomes hollow establishing the gastrovascular cavity. The tissue which has been pushed inward around the "yolk plug" is now



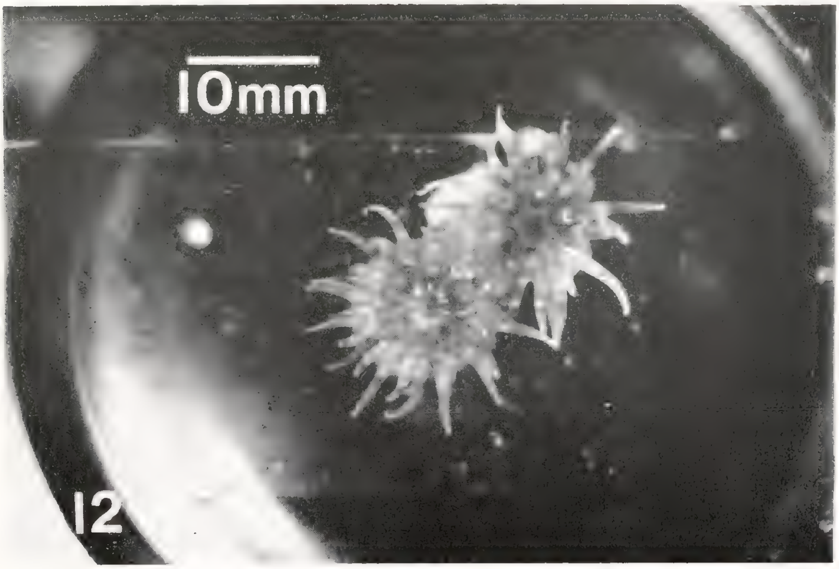


FIGURE 11. Young anemone with 8–12 tentacles, one month after settlement on the *Phyllochaetopterus* tubes.

FIGURE 12. One year old anemone when fed in the laboratory.

the pharynx (Fig. 20). Eight primary septa begin to develop simultaneously, but there is as yet no sign of development of tentacles.

The larva is now generally sticky and can temporarily attach to the surfaces of the culture dishes. Although secretory cells and nematocysts are found distributed throughout the ectoderm, planula larvae were seen only to make contact on the substratum with the apical end. It is difficult at this stage to pick them up without their sticking to the pipette. They are now nine days old and presumably ready to settle. The chronological development is summarized in Table I.

Effect of substrata on larval settlement

To test the effect of substratum on larval settlement, we set up four finger bowls, each with 150 ml of sea water, and added: (a). *Phyllochaetopterus* sp. (a polychaete worm) tubes, a substratum which has been effective in inducing the larvae of the starfish, *Mediaster aequalis,* to metamorphose (Birkeland, Chia and Strathmann, 1971); (b). some tubes of *Sabellaria cementaria,* another polychaete worm, a substratum used by Long (1964) to induce brachiopod larvae metamorphosis; (c). three slides coated with wax known to induce ascidian larvae to metamorphose, along with a few small stones; and (d). more sea water to serve as control. In each dish we placed 20 larvae from the same spawn which were 10 days old. All of those in dish (a) settled within three days; of those in dish (b), only 11 settled in three days, the rest settled within 10 days; and those in dishes (c) and (d) did not begin to settle until 17 days later, but eventually all settled within the second month. Thus, both *Phyllochaetopterus* and *Sabellaria* tubes can induce the settlement of planula larvae; the *Phyllochaetopterus* being more effective. But the presence of such a substratum is not essential, since planulae in glass dishes will also settle, although after a delay of 17 days. The settlement on these worm tubes is not permanent; they can detach themselves from the substratum and resettle on the glass of the culture dishes.

TABLE I

Chronology of larval development

Time (Days)	Developmental events
1	Fertilization; superficial cleavage up to 16-cell stage
2	Blastula; spiny membrane evident
3	Gastrula; formation of "yolk plug"; ciliated; free swimming; spiny membrance still evident
4–5	Gastrula; "yolk plug" disappears; blastopore as a small indentation; spiny membrane disappears.
6	Post-gastrula or young planula; elongated, 500 × 750 μ in size; blastopore becoming the mouth; apical organ formed by invagination
7–9	Planula, more elongated
10	Planula; becoming sticky; begin to make contact with substratum; able to change shape
11	Settlement when *Phyllochaetopterus* or *Sabellaria* tubes are present; newly settled larvae, when extended, measure: diameter of pedal disk = 0.6 mm, length of column = 0.8 mm; 8 septa appeared
17	Young anemone with 4 tentacles
21	Young anemone with 8 tentacles; body wall translucent white; begins to feed
27	Settlement in dishes with sea water only or with small stones and wax-coated slides

The first 4 tentacles begin to appear 7 days after settlement and the second 4 tentacles appear 5 days later. At this time, when the young anemones have had 8 tentacles, they begin to react to food such as *Artemia,* mussel or clam meat. The body wall is translucent and whitish in color. The young anemones when fully extended average 0.6 mm in diameter and 0.8 mm in height, and the tentacles are 1 mm long (Fig. 11).

It is interesting to note that the planula larvae which are not settled in dishes (c) and (d) begin to develop tentacles when they are 20 days old; this is only a 3-day delay compared with those settled on worm tubes in dishes (a) and (b). It seems that although the larval settlement is greatly enhanced by environmental factors, such as substratum, the development of tentacles is more or less fixed in a certain age and independent of settling.

Growth of the juvenile anemones

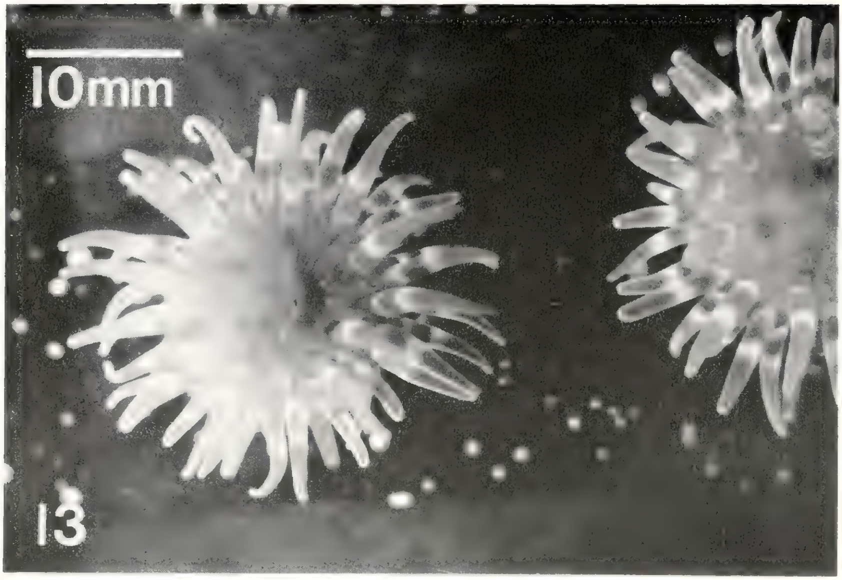
At two months old the anemones measured 0.8 mm across the oral disc and had 12 tentacles averaging 1 mm in length. By the beginning of the fifth month the diameter of the oral disc had increased to an average of 1.7 mm and the anemones had 12 to 16 tentacles averaging 2.1 mm in length. At this time some of the juvenile anemones were transferred to glass slides and put into the "Plankton-Kreisel." Both groups of anemones were fed *Artemia* biweekly until the end of the fifth month. From the beginning of the sixth month the anemones in the "Plankton-Kreisel" were fed mussel or shrimp meat once every month and after the tenth month the feeding was increased to weekly. On the other hand, the anemones in the culture dishes received periodic changes of water but no food. During the tenth month the fed anemones in the "Kreisel" began to develop dark colored bands on their tentacles and oral discs, and their columns became orange in color. The non-fed anemones in the culture dishes did not change.

At one year of age the large fed anemones had reached 10.0 mm in diameter across the oral disc and had 30 to 35 tentacles averaging 6.5 mm in length (Fig. 12). At 14 months of age those anemones that had been fed reached 15 mm in diameter and had 45 to 50 tentacles which averaged 7 mm long (Fig. 13). The column had the red patching common on the adults and the verrucae on the upper margins of the column developed white coloration. The starved ones, by contrast, had not developed the adult coloration and were still translucent white, but with an opaque white band on the tentacles; they still had only 12 to 16 tentacles; the oral discs averaged 1.2 mm in diameter (Fig. 14). In other words, they had not grown during nine months of starvation; in fact, their size had reduced.

At this writing the anemones are 18 months old. Those that have been fed have reached 4 cm in diameter with 60 to 70 tentacles. A few of the starved anemones are still alive and have not changed size appreciably.

DISCUSSION

The identification of the various species in the genus *Tealia* is still problematic. Based on some unpublished studies of the cnidomes of the anemones of the Friday Harbor region (McIntyre, 1960, unpublished class report), we are reasonably certain in our identification. It might be useful, however, to describe briefly the animals we have studied and hopefully our observations on the development may help to elucidate the taxonomic problems. The anemone is found attached to rocks



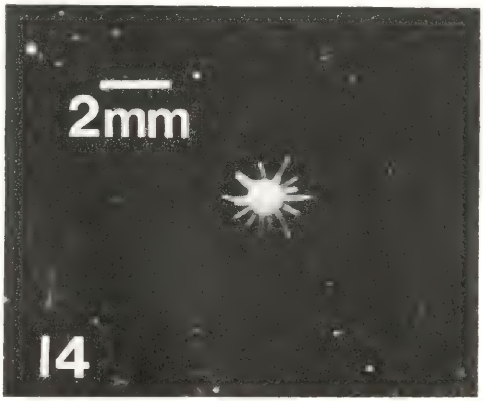


FIGURE 13. 14 month old anemone when fed in the laboratory.
FIGURE 14. 14 month old anemone, when starved for the last 9 months.

in the low intertidal and subtidal. Its base is broad (10 to 15 cm in diameter) and strongly adherent. The column is stout and cylindrical. The color varies from a yellow-brown to green and may have irregular red patches. Weakly developed verrucae may cover the column in regular rows, and are generally the same color as the column. In some specimens the verrucae on the upper margin of the column are white. No shelly or rocky materials are found adhering to the verrucae. The tentacles are short and blunt, usually light in color with one white or red band. The oral disc is usually a lighter color than the column. Red to brown lines often surround the bases of the tentacles.

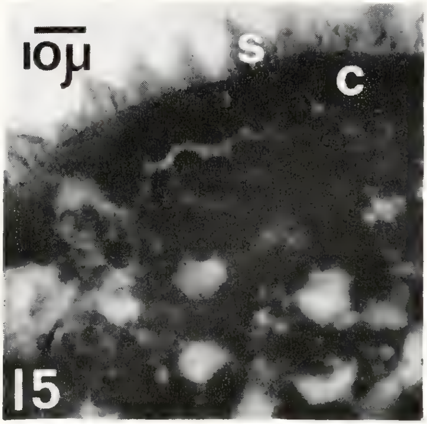
The spiny egg membrane of *Tealia crassicornis* is similar to that described in other anemones. They are known to occur on the eggs of *Peachia* (Faurot, 1895), *Bolocera* and *Actinia* (Gemmill, 1920, 1921) *Anthopleura, Cribrinopsis* and *Actinostola* (unpublished observations). The eggs of some other anemones are reported to have smooth membranes: *Halcampa* (Nyholm, 1949), *Metridium, Adamsia* and *Sagartia* (Gemmill, 1920). These reports of smooth membranes need verification in light of the following experience. Our first observations of the egg of *Actinostola* using bright field optics led us to call it a smooth membrane. Using phase contrast optics we observed this egg and found it to be covered by very fine spines. The spines are unique in the eggs of actiniarians. Their function is at present unknown.

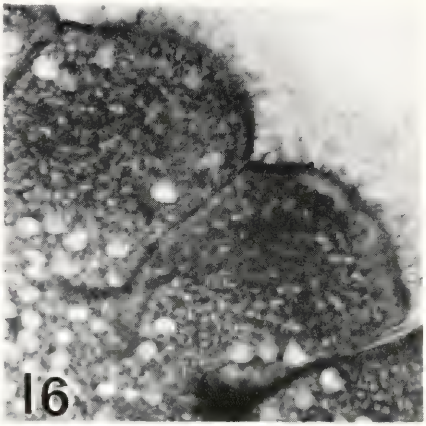
Appellöf (1900) studied the embryology of *T. crassicornis* and his description of the development prior to gastrulation agrees with ours. The major difference between our interpretation and that of Appellöf is the process of germ layer formation. He described and figured an invagination process in which the central yolky material passed between the invaginated cells and came to lie in the gastrovascular cavity. The same mechanism has been reported in *Bolocera tuediae* by Gemmill (1921) and in *Actinia bermudensis* by Cary (1910). Our observations indicate that the endoderm is likely established by multipolar ingression. These cells that are moved into the interior do not form a well defined layer of endoderm until much later in development.

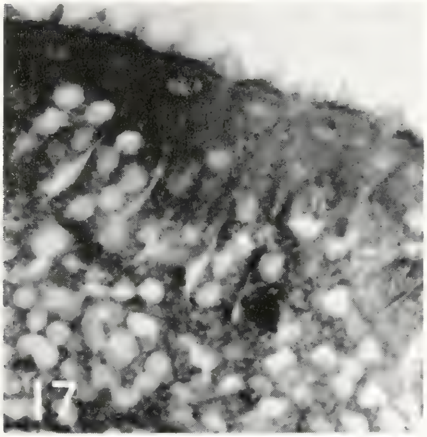
The formation of the so called "yolk plug" by epiboly has apparently escaped the attention of Appellöf (1900). Our study indicated that cells at the animal half proliferate rapidly and move towards the vegetal pole where cells have stopped dividing. Thus, a number of large, nondividing cells, the "yolk plug," are being overgrown and eventually are enclosed inside the embryo. The opening left from the "yolk plug" is the blastopore and later the mouth. The gastrovascular cavity (archenteron) was filled by yolk material throughout the embryonic development; it became hollow only at a much later stage (planula) by the gradual absorption of yolk.

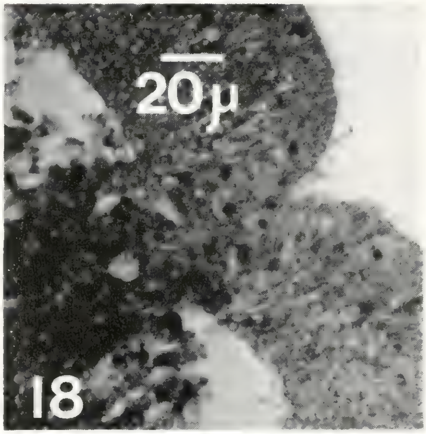
The apical invagination described in this paper was not reported by Appellöf (1900). There is little doubt that the invaginated cells will be incorporated in the apical organ, although our histological sections of the planula larva show that the apical cells differ only slightly from other endodermal cells. Widersten (1965) did not find an apical organ in *Tealia felina*. He made the point that the apical organ is functionally important in the feeding of the larva and during the evolutionary changes from planktotrophic larvae to lecithotrophic larvae, in the phylum *Cnidaria,* the apical organ has been lost. Judging from the fact that the larvae of *T. crassicornis* will respond to different substrata and alter their time of settlement, we feel that the primary function of the apical organ is substratum selection, not feeding. Therefore, it is not surprising that the larvae of *T. crassicornis,* though lecithotrophic, possess an apical organ.

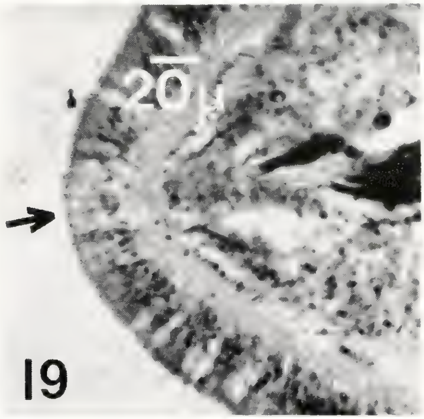
The effect of the worm tubes in promoting the settlement of larvae is not restricted to the planulae of *Tealia.* The larvae of *Cribrinopsis,* another sea anemone, respond in a similar manner. In the echinoderms, the larvae of the asteroids *Mediaster aequalis* and *Pteraster tesselatus,* and of the holothuroids *Cucumaria miniata* and *Psolus chitonoides* can be induced to settle in the presence of *Phyllochaetopterus* tubes as well (Fu-Shiang Chia, unpublished observations). The nature of the interaction is, however, unknown.











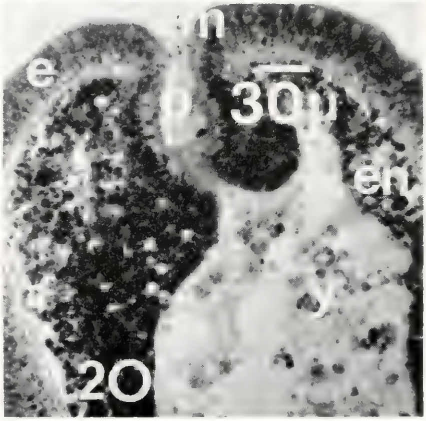

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FIGURE 15. Section of a fertilized egg, showing the surface spines (s) and cortical cytoplasm (c).

FIGURE 16. Section of an early cleaving embryo, showing the incomplete cell membrane.

The growth of the juvenile, as is shown in our study, is totally dependent upon the amount of food it takes, and hence the size of the animal has little to do with age. It is interesting to note that the young anemones are able to withstand at least nine months and possibly a much longer period of starvation. The starved animal not only did not grow but decreased in size. During this period they may be directly utilizing dissolved organic molecules from sea water or they may feed on micro-organisms for maintaining themselves.

Based on the differences of the growth rate between well-fed and starved animals as we have observed, it is impossible to speculate upon the age of a typical reproductive adult of *T. crassicornis* (10 to 15 cm in diameter). It is certain, however, that more than one year is required to reach the reproductive condition since our well-fed specimens show no sign of gonad development at 14 months.

The fact that *T. crassicornis* are brooders in some areas and shed gametes freely into the sea at Friday Harbor may either be due to the fact that the two populations are two different species, or that they are the same species but differ in reproductive behavior. The plasticity in the mode of reproduction exists also in other sea anemones such as *Actinia equina* as discussed by Chia and Rustron (1970), and *Sagartia troglodytes* discussed by Stephenson (1929).

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SUMMARY

1. *Tealia crassicornis* in the Friday Harbor Laboratories was observed to spawn from April to June. The eggs measure 500–700 μ in diameter, and bear surface spines 25 μ in length.

2. The cleavage is superficial, resulting in small cells on the animal pole and large cells on the vegetal pole.

3. Endoderm appears to be formed by multipolar ingression while the central yolk mass remains uncleaved and the formation of the gastrovascular cavity is achieved by absorption of yolk material.

4. The settlement of planula larvae can be facilitated by adding the tubes of the polychaete worms, *Phyllochaetopterus* or *Sabellaria* to the culture dishes.

5. A chronology of development is presented and the histogenesis of larval tissue is described.

FIGURE 17. Section of a late cleaving embryo, showing the superficial layer of cells and the uncleaved yolk mass.

FIGURE 18. Formation of apical organ by invagination of ectoderm at the apical end of a gastrula, the space between the ectoderm and the endoderm and yolk mass in this photograph is an artifact.

FIGURE 19. Sagittal section of a planula larva showing the apical group of cells with clear cytoplasm (arrow).

FIGURE 20. Sagittal section of a planula larva showing the mouth (m), pharynx (p), ectoderm (e), endoderm (en), and the remnant of the yolk mass (y).

6. The young anemones have grown to a size of 4 cm in diameter with 60 to 70 tentacles within 18 months when fed in the laboratory.

7. Young anemones can withstand starvation for at least 9 months. There is no growth when starved.

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THE ROLE OF ULTRASTRUCTURE AND PHYSIOLOGICAL DIFFERENTIATION OF EPITHELIA IN AMINO ACID UPTAKE BY THE BLOODWORM, *GLYCERA*

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Soft-bodied marine invertebrates are able to remove amino acids and other small organic compounds from very dilute solution in the surrounding sea water. The evidence for the occurrence and potential significance of this phenomenon has been recently reviewed (Stephens, 1967, 1968, in press). It has been shown in a number of cases that uptake of amino acids can occur directly across the body wall. This has been demonstrated by mechanically occluding the gut (Stephens, 1962, 1963, 1964; Chapman and Taylor, 1968) and can also be concluded from autoradiographic studies (Ferguson, 1967; Pequignat and Pujol, 1968; Little and Gupta, 1969).

In most invertebrates studied, as the concentration of amino acids is increased in the ambient medium, the rate of uptake also increases provided concentrations are low. At higher concentrations, the system mediating uptake becomes saturated and further increase in ambient concentration does not produce a corresponding increase in the rate of uptake. This system can be described adequately by Michaelis-Menten kinetics. The maximum velocity of uptake (V_{max}) and the ambient concentration at which the system is half-saturated (K_t) can be determined. The reported K_t values for most marine invertebrates ranges from 5×10^{-5} to 2×10^{-3} moles liter and that of V_{max} from 10^{-7} to 10^{-5} moles/g/hour (Stephens, in press). Southward and Southward (1970) determined the K_t value for certain Pogonophora to be as low as 10^{-7} moles/liter.

The bloodworm, *Glycera dibranchiata*, has been shown to accumulate amino acids and creatine from the surrounding medium (Stephens, Van Pilsum and Taylor, 1965). Preston and Stephens (1969) and Preston (1970) have shown that coelomocytes of *Glycera* suspended in sea water concentrate amino acids very rapidly. Thus both the worm as an organism and at least some of its tissues have this capacity. Uptake by coelomocytes is correlated with the maintenance of large pools of free amino acids intracellularly and is presumably the mechanism whereby large differences between cells and extracellular fluid are achieved.

Glycera is a favorable animal for a further analysis of the pathway (or pathways) of entry of dissolved organic compounds. It is a large worm. The gut is suspended in an extensive, undivided coelom. Circulation of the hemoglobin-containing coelomocytes is accomplished by ciliary currents and there is no other circulatory system. Its comparatively simple organization and size facilitate experimental analysis. In particular, it is easy to obtain portions of the body wall free of other tissues for study. Klawe and Dickie (1957), and Clark (1962), give an account of the morphology and general biology of the worm. This paper is a report of the structure of the external and internal epithelia of the body wall and the way they contribute to the uptake of amino acids from the surrounding sea water and the coelomic fluid.

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MATERIALS AND METHODS

Animals were commercially collected in Maine and shipped by air to California. We purchased worms from local dealers as needed and maintained them in sea water at 10–15° C. In some cases, worms were maintained in water or in moist seaweed at lower temperatures.

For general histology and orientation, portions of worms were fixed in Bouin's solution and embedded in paraffin. Sections were stained with paraldehyde fuchsin and picro-indigo carmine (Gabe, 1953).

For electron microscopy, small pieces of body wall were fixed in 5% glutaraldehyde and postfixed in 2% osmium tetraoxide. Both fixatives were buffered to pH 7.2 by 0.1 molar phosphate buffer with or without the addition of 0.45 molar sucrose. Tissues were then dehydrated in an acetone series and embedded in Epon 812 (Luft, 1961).

Glass or diamond knives were used to cut sections on a Reichert OMU2 ultramicrotome. 1 to 2 μm sections stained with toluidine blue were studied under a light or phase microscope. Contrast of thin sections was enhanced by Karnovsky's (1961) and Reynolds' (1963) lead stain or by 2% phosphotungstic acid. Most satisfactory results were obtained with 2% aqueous uranyl acetate followed by Reynolds' lead citrate.

Electron micrographs were taken with a Zeiss EM 9A or 9S-2 microscope.

Worms prepared for scanning electron microscopy were quick frozen in isopentane chilled with liquid nitrogen and then freeze-dried by the moving gas method (Jensen, 1962). Dried tissue was coated with gold and carbon in a Varian vacuum evaporator, and examined in JEOL scanning electron microscope.

Morphology and amino acid composition of isolated cuticle was also studied. Large pieces of isolated cuticle were mounted on glass slides, air dried, and observed under a phase microscope. Worms were anesthetized by adding ethanol slowly to the sea water or using ether (Reed and Rudall, 1948). Cuticle was stripped from the body using fine forceps. The cuticle was then washed repeatedly in distilled water and kept overnight at 4° C in distilled water to remove adhering epithelial cells. Samples were checked by electron microscopy to ensure the absence of adhering cells. For determination of amino acid composition, the cuticle was hydrolysed in a sealed vial at 100° C in 6 N HCl. The HCl was evaporated off and the amino acid residues dissolved in a small amount of 80% ethanol. This solution was spotted on a cellulose thin-layer plate (20 × 20 cm MN-Polygram Cel #300, Brinkman Instruments, Inc.). The amino acids were separated two-dimensionally using the solvent systems described by Jones and Heathcote (1966). The plates were air dried and sprayed with 2% ninhydrin in absolute ethanol. Color spots were developed at room temperature in darkness (Clark, 1968).

C^{14}-labelled amino acids were used in the uptake studies. Concentrations of amino acid greater than 10^{-6} molar were obtained by adding C^{14} amino acids as required. Whole worms were exposed to the labelled solutions in beakers. Isolated pieces of body wall were exposed as described below. All experiments were run at room temperature (21 ± 1° C). Both disappearance of radioactivity from the medium and appearance of radioactivity in the tissue were followed when possible. After a suitable time of exposure, whole worm or isolated pieces of body wall were washed in sea water and extracted with 80% ethanol or 5% cold trichloroacetic acid. Duplicate samples of sea water or tissue extract were mixed with 10 ml of a scintillation cocktail (2 volumes of a solution of 6 g PPO/liter of toluene and 1 volume of a detergent, Triton X-100) and counted in a Beckman CPM-100 scintillation system. Some of the samples from whole worm uptake experiments were dried on aluminum planchets and counted with a thin-window gas flow GM detector. All data are corrected as necessary for quenching and background.

Uptake of amino acids across the external and the internal surface of the body wall was examined using a Ussing chamber. The cross-section area of the windows through which the tissue was exposed to the solution in the two halves of the chamber was approximately 0.5 cm^2. Solution volume was 10 ml on each side. Pieces of body wall approximately 2 × 3 cm were isolated, rinsed in sea water and clamped between the halves of the chamber. C^{14}-labelled amino acid at a concentration of approximately 10^{-6} moles per liter (1 microcurie in 20 ml) was supplied on one side and sea water bathed the other. Radioactivity of an 80% ethanol extract of the tissue was determined after a suitable exposure time.

A simplified smaller chamber (Fig. 1A) consisting of a tube open at both ends with a broad base was used in further studies on the uptake kinetics of the external and the internal surface. The cross-section area of the chamber was approximately 0.6 cm^2. Volume of solutions used was reduced to 0.5 ml or less. Thus, radioactivity disappearing from the medium as well as that appearing in the tissue could be measured. A similar chamber (Fig. 1B) was modified by closing the top and providing entrance and exit ports for study of uptake for longer time periods.

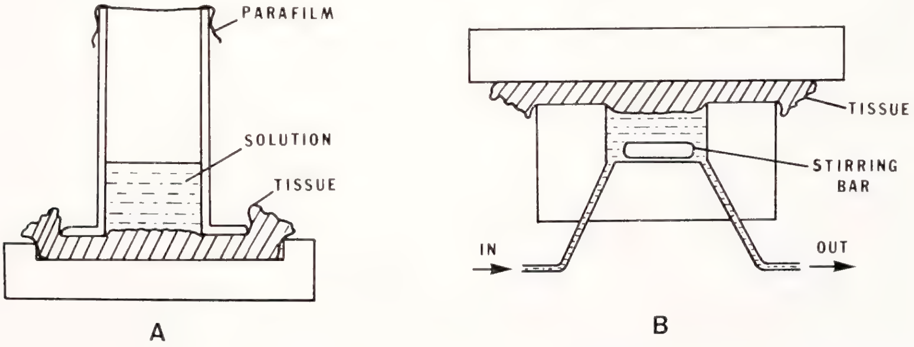
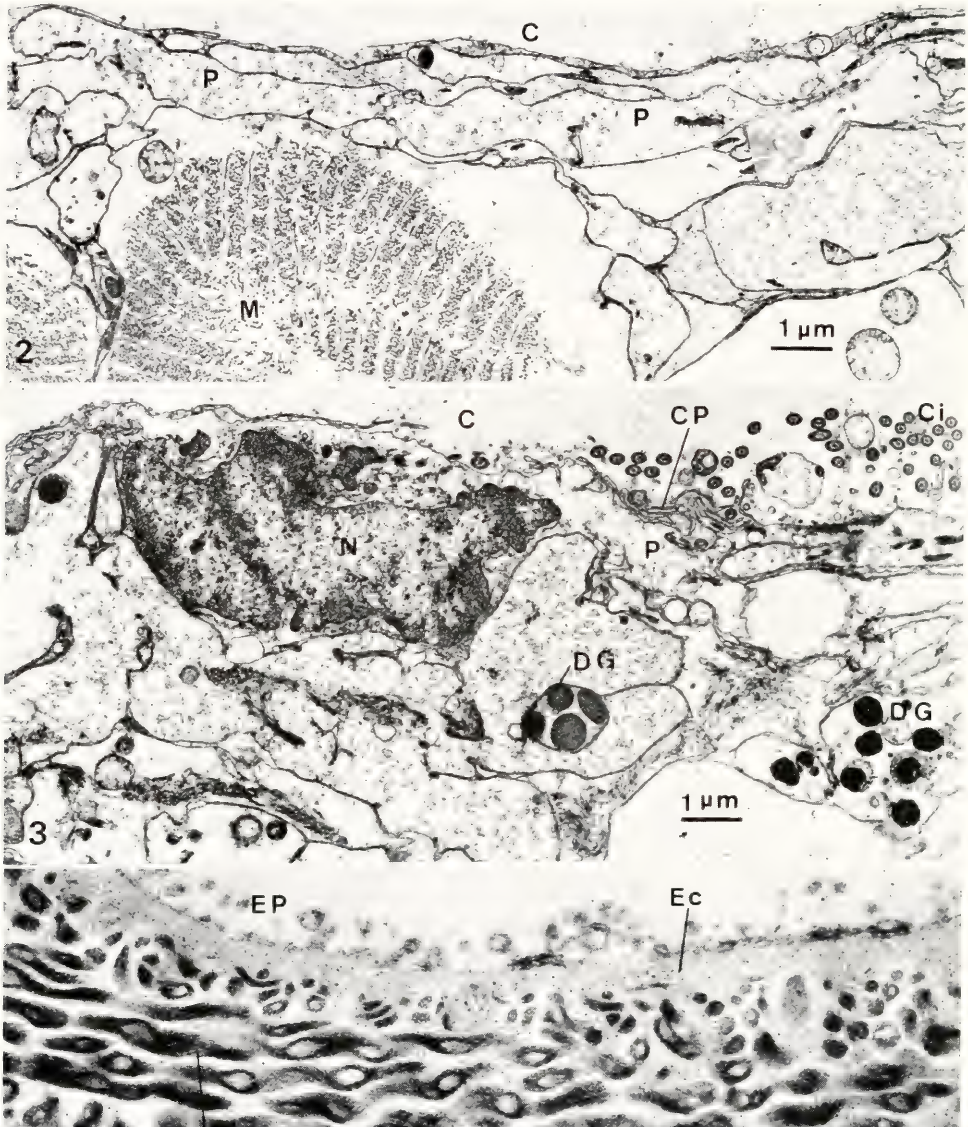


FIGURE 1. (A) Small chamber used in studies on the kinetics of amino acid uptake by the body wall of *Glycera*. Mixing of solution was achieved by mounting this preparation on a shaker. (B) Closed chamber used in studies on continuous uptake. Amino acid solution was passed through the chamber by a peristaltic pump and effluent solution was collected by a fraction collector. The small magnetic stirring bar was used for mixing in the chamber.


C
P
P
M
1 µm
2
C
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DG
DG
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3
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Mv
Mv
CT
1 µm
4

Figures 2–4.

RESULTS

A. *Light microscopy*

The body wall of *Glycera* consists of two epithelial and two muscle layers. The outer, circular muscles form a complete ring around the inner, longitudinal muscles. The longitudinal muscles are arranged in four massive bundles due to the presence of the lateral parapodia, the dorsal septa and the ventral nerve cord. The peritoneum lines the coelomic surface of the longitudinal muscles. The epidermis attaches to the outside of the circular muscles by a basement membrane. It consists of columnar to pseudo-stratified columnar cells. A large number of goblet cells is scattered among the apical portion of the columnar cells. The contents of the goblet cells stain purple with paraldehyde-fuchsin which indicates the presence of mucopolysaccharides. The epidermis is covered by a non-cellular cuticle. Under the phase microscope, the cuticle has a laminated appearance. It stains blue with paraldehyde-fuchsin, suggesting the presence of collagen (Gabe, 1953).

Isolated cuticle, dried on glass slides, shows layers of fibers crossing at right angles. All fibers form a 45° angle with the long axis of the worm. Refractile tubules, interpreted as openings of the goblet cells, perforate the network of fibers at regular intervals.

B. *Electron microscopy*

The peritoneum which covers the coelomic surface of the longitudinal muscles, is composed of one to several layers of flattened cells (Fig. 2). In certain areas, ciliary bands and irregular cytoplasmic projections extend into the coelom (Fig. 3). In these cells, large membrane-bound electron-dense granules up to 1 μm in diameter are common. Mitochondria, Golgi bodies, endoplasmic reticulum and other organelles are rarely seen. In general, these epithelial cells have a very simple organization.

The structure of the epidermis and cuticle is much more complicated (Fig. 5). The cuticle ranges from 1 to 12 μm in thickness. It is thinnest over the parapodia and gills. The fibers observed and described at the light microscope level, appear as layers of highly ordered and densely packed tubules in electron micrographs (Figs. 4 and 6). Each layer is parallel to the surface but oriented perpendicularly to adjacent layers. Fifteen or more layers may occur (Figs. 5 and 6). Individual tubules measures 0.1 to 0.3 μm in diameter and are composed of about 5 nm thick fibers. The largest tubules occur in central layers where the lumen (or electron-transparent region) is clearly shown (Fig. 6). The tubules in the innermost and outer layers are smaller and look solid at times. Two per cent phosphotungstic acid provides optimum contrast and shows the tubular nature best (Fig. 4). A homogeneous epicuticular layer lies over the network of tubules, which in turn is

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FIGURE 2. A typical view of the coelomic lining of the muscular body wall of *Glycera dibranchiata;* C = coelom; M = muscle; P = peritoneum; Uranyl acetate and lead citrate stain.

FIGURE 3. Portion of coelomic lining showing irregular cytoplasmic extensions (CP) and cilia (Ci); C = coelom; DG = dense granule; N = nucleus; P = peritoneum; Uranyl acetate and lead citrate stain.

FIGURE 4. Apical portion of *Glycera* cuticle stained with 2% phosphotungstic acid to show the tubular nature of the collagen tubules (CT) and the microfibers (Mf); Ec = Epicuticle; EP = epicuticular particles; Mv = microvilli.

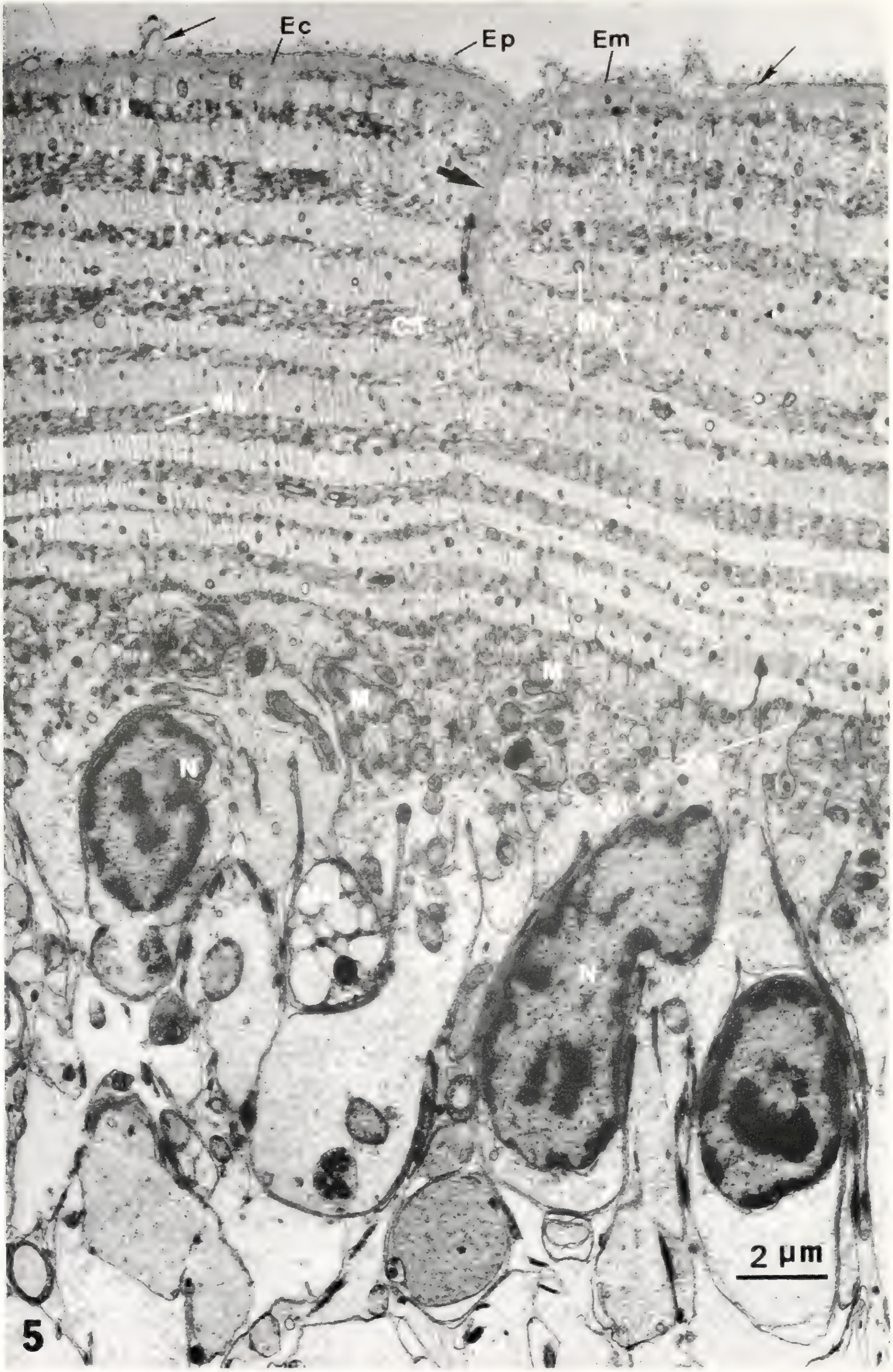
Ec
Ep
Em
Ct
My
M
M
M
M
N
N
2 μm
5

FIGURE 5.

outlined by an electron-dense epicuticular membrane (Fig. 5). Dense epicuticular particles about 0.1 to 0.2 μm in diameter stud the surface (Figs. 4, 5 and 6). The goblet cells are seen at various stages of secretion. The neck of each is surrounded by an invagination of the epicuticle (Fig. 5), which is seen as a refractile tubule in the light microscope. A large number of cytoplasmic extensions from the apical end of the columnar epithelial cells invades the cuticle proper (Fig. 7). Occasionally, they reach the surface but more typically terminate in the cuticle. These cytoplasmic extensions or microvilli measure up to 0.5 μm in diameter. Higher magnifications show the presence of particles about the size of free ribosomes in the microvilli, and bundles of tonofilaments at the more electron dense base. Mitochondria, endoplasmic reticulum, Golgi bodies, vesicles of various sizes and even nuclei occur at the apical portion of these columnar cells. In this region cells interdigitate with one another (Fig. 5). Zonula adherens from 0.2 to 0.5 μm long join the lateral sides of contiguous cells close to the cuticle. Electron dense striations occur in the cell junction below the zonula adherens which suggests the presence of septate desmosomes.

Scanning electron microscopy confirms the observations on the epicuticular particles, gland openings and the presence of some mucus material seen with the transmission electron microscope. Microorganisms have not been observed adhering to the general surface of *Glycera* by either method.

The increase of surface area due to the presence of microvilli was estimated by measuring the diameters or the width and lengths of the microvilli found in typical sections. The total circumference calculated was used as an index of their surface area. The width of epithelial cells was considered to be a measure of the surface area if the microvilli were absent. A comparison between the external and the internal surface was made. The relative measurements are expressed in arbitrary units in Table I. This estimation is only a rough one because of the complexity of the microvilli and the irregularity of the membrane foldings. As an average, the epidermal microvilli increase the surface area about thirteen times. The membrane foldings on the internal surface roughly double the surface area. Therefore, the epidermal surface has a total area about seven times that of the internal surface.

C. *Amino acid composition of the cuticle*

The general separation of amino acid residues of the cuticle hydrolysate was very good on the thin-layer chromatograms, but arginine did not separate completely from lysine and the glycine spot overlays hydroxyproline at high concentrations. A total of eighteen amino acids were identified from the chromatograms. Amino acids present in largest quantities were hyroxyproline, glutamic acid, lysine, glycine, alanine, and arginine. Proline, serine, leucine, threonine, valine, cystine and isoleucine were present in fairly high concentrations while

Figure 5. Cuticle and apical portions of *Glycera* epidermal cells. The numerous microvilli (Mv) penetrate the orthogonal layers of collagen tubules (CT) and occasionally come to the external surface (arrows). Note the concentration of organelles in the columnar cells (CC), the tangential section of a mucus cell (Mu) and part of its duct (arrow head). The duct is seen only as the invagination of the epicuticle (Ec) here; EM = epicuticular membrane; EP = epicuticular particles; M = mitochondron; ZA = zonular adherence; Karnovsky's stain.

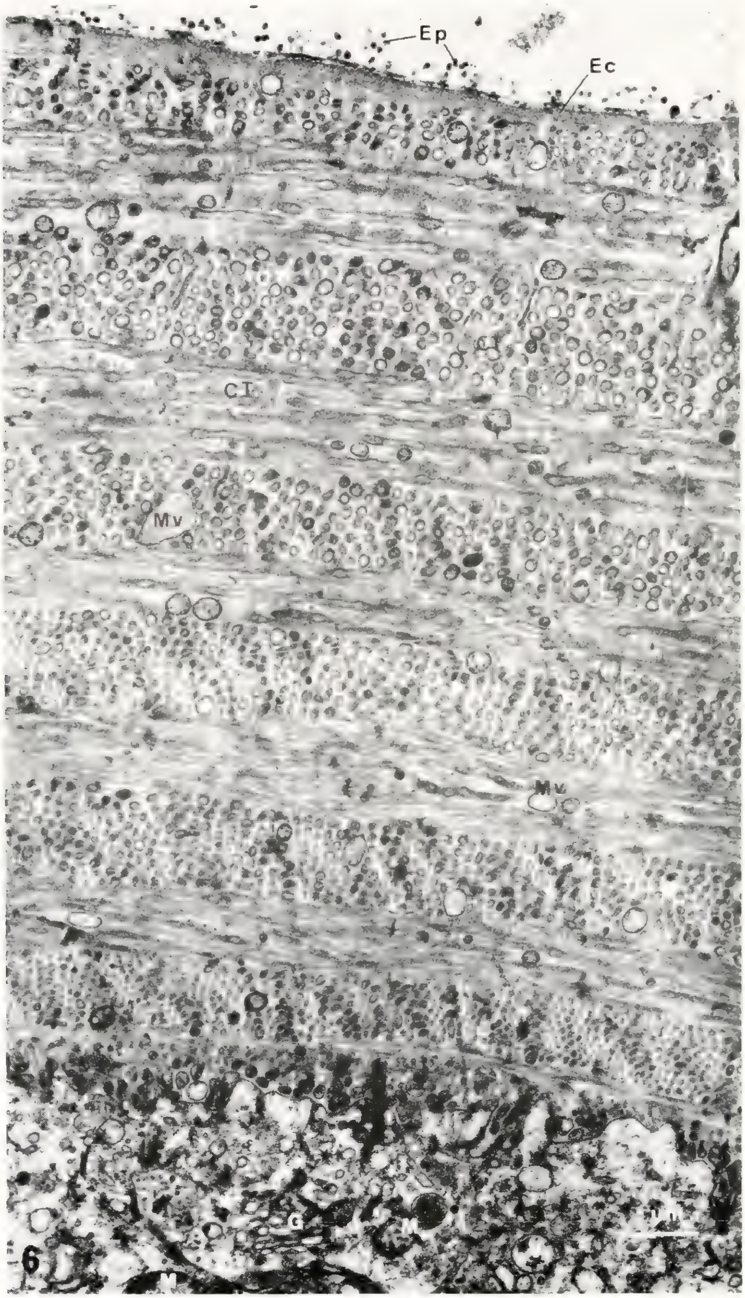

FIGURE 6. Orthogonal collagen tubules (CT) seen at longitudinal and cross sections. Note the diameter of the tubules are slightly smaller in those layers close to the epidermis and the epicuticle (Ec). Golgi apparatus (G), mitochondria (M), vesicles (V) and other organelles are found in the epidermal cells; Uranyl acetate and lead citrate stain.

TABLE I

Relative increase in surface area due to the presence of cytoplasmic extensions on the external and internal surface of Glycera *body wall (arbitrary units)*

Surface area of microvilli	Surface area of cells	Ratio	Average ratio
External surface			
2680	250	10.7	
1970	120	16.4	
1790	180	10.0	12.8
1680	100	16.8	
1020	100	10.2	
Internal surface			
120	150	0.8	
29	230	0.1	
0	200	0.0	0.82
22	200	0.1	
565	180	3.1	

phenylalanine, tyrosine, methionine, aspartic acid and histidine were present only in traces.

D. *Amino acid uptake*

Whole worms. When *Glycera* was placed in a solution of C^{14}-labelled amino acid, radioactivity decreased steadily in the medium and appeared in the worm. This observation was previously reported for uniformly labelled glycine and arginine (Stephens, Van Pilsum and Taylor, 1965). The present observations include six other amino acids. Rates of uptake were measured at concentrations ranging from 10^{-5} to 10^{-3} moles/liter of amino acid. The K_t values listed in Table II were obtained graphically using the Lineweaver-Burk linear transformation of the Michaelis-Menten equation.

After whole blood worms had been exposed for one hour to C^{14}-labelled non-metabolizable amino acid analogs, 1-aminocyclopentane-1 carboxylic acid and α-aminoisobutyric acid, about 1% of the radioactivity which disappeared from the medium was recovered from the gut. Only 5% was located in the eversible

TABLE II

Kinetics of amino acid accumulation in whole worms, Glycera dibranchiata

	K_t – Molar	V_{max} – moles/hr/g worm
Alanine	2.08×10^{-3}	9.62×10^{-7}
Arginine	2.17×10^{-4}	1.61×10^{-7}
Aspartic acid	2.30×10^{-3}	1.56×10^{-7}
Glycine	2.52×10^{-4}	2.04×10^{-7}
Valine	1.20×10^{-3}	1.64×10^{-7}
Proline	3.68×10^{-4}	1.06×10^{-6}
Glutamic acid	3.93×10^{-4}	—
Phenylalanine	2.01×10^{-4}	—

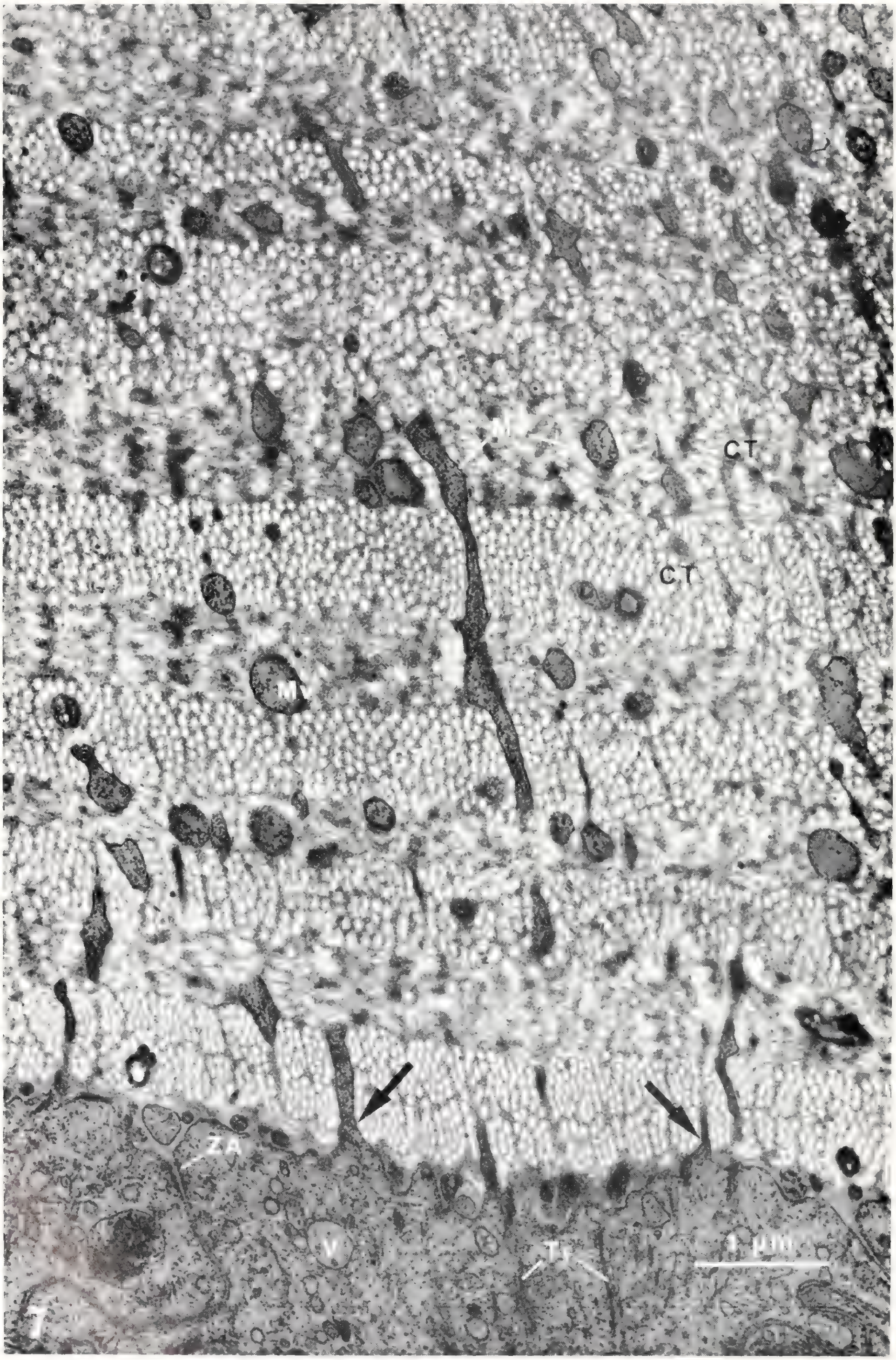
M
CT
CT
M
ZA
V
Tr
1 µm
7

FIGURE 7.

proboscis and more than 75% was in the body wall. Thus, influx of amino acids occurs principally through the general body surface under these experimental conditions.

Isolated body wall. When isolated portions of the muscular body wall of *Glycera* are clamped into the Ussing chamber as indicated in Figure 1, C^{14}-labelled amino acids enter more rapidly across the external surface than the internal surface. Table III lists rates for glycine and phenylalanine, uptake across the external surface being markedly more rapid in both cases (13: 1 and 7: 1, respectively). After a fifteen minute exposure time, no detectable radioactivity was transferred from either chamber to the other. In fact, other observations continued for periods as long as 210 minutes showed no transfer of label.

Continuous uptake of amino acids was also followed. Pieces of body wall were clamped into a modified chamber such that only one surface of the tissue was exposed at a time. Amino acid solution was passed through the chamber at a constant rate by using a peristaltic pump. A small magnetic stirrer was used for mixing in the chamber. The effluent solution was collected by a fraction collector and radioactivity was determined. Both surfaces of the isolated body wall were found to remove labelled amino acids at a constant rate over a period of three hours. Antibiotics were used in these prolonged observations to prevent bacterial contamination.

TABLE III

CPM in an alcohol extract of the body wall of Glycera *after exposure to 10^{-5} molar glycine-C^{14} or L-phenylalanine-C^{14} supplied to the internal or the external surface*

	External	Internal
Glycine	1580	125
	1350	65
	1000	105
Phenylalanine	530	82
	1040	141
	890	131

Kinetic parameters of the internal and external body wall surfaces were studied employing the simplified, small-volume chambers and using the nonmetabolizable amino acid, taurine. Taurine uptake by the external surface of isolated body wall shows kinetics similar to that of the whole worm (Fig. 8). K_t and V_{max} were evaluated using the Hofstee linear transformation (Dowd and Riggs 1965). The K_t has a value of $(6.3 \pm 1.7) \times 10^{-4}$ moles/liter. The V_{max} is $(8.53 \pm 1.44) \times 10^{-8}$ moles/hr/cm^2 (Fig. 9). The internal surface, however, behaves quite differently. The system takes up taurine but shows no sign of saturation at concentrations as high as 5×10^{-3} moles/liter. When the rate of uptake is plotted against medium concentration, a straight line passing through the origin is obtained (Fig. 8). A

FIGURE 7. Favorable section showing the microvilli (Mv) extending from the epidermal cells into the cuticle proper (arrows). Tonofilaments (Tf), zonular adherence (ZA) and various vesicles (V) can be seen; Karnovsky's stain.

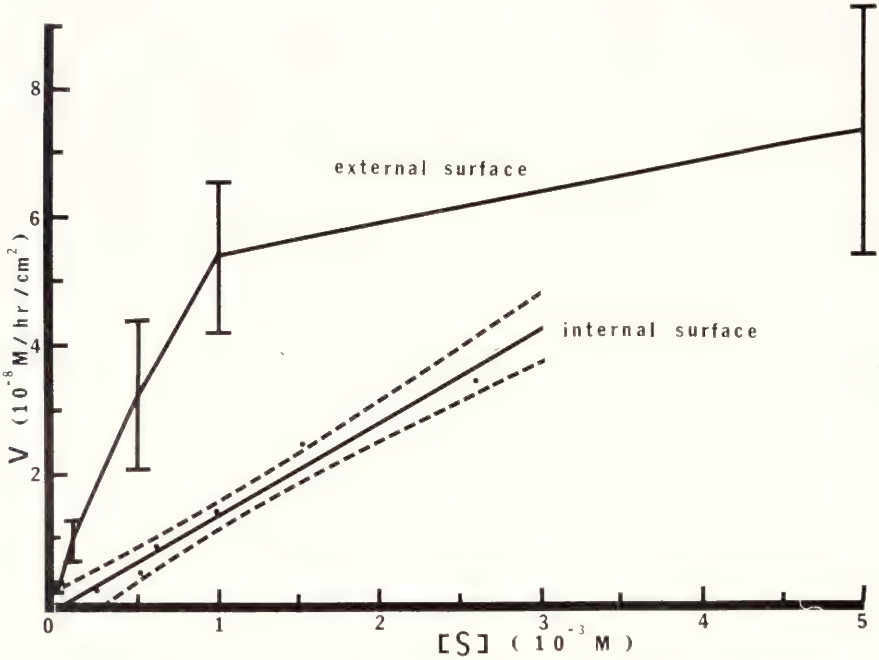

FIGURE 8. Velocity of taurine uptake by isolated pieces of *Glycera* body wall as a function of the medium concentration. The vertical bars indicate standard deviations. The broken lines show the 95% confidence interval of the linear regression calculated by the least squares method.

Hofstee plot results in a straight vertical line, which does not intercept the ordinate (Fig. 9). The rate of uptake is much lower than that shown by the external surface in agreement with the observations on glycine and phenylalanine reported above.

DISCUSSION

The general organization of the body wall of *Glycera* is typical of that of errant polychaetes. The ultrastructure of the peritoneum is similar to that which lines the coelomic surface of the proventriculus of *Syllis* (Boilly, 1970). The cuticle of *Glycera* stains blue with paraldehyde-fuchsin which suggests the presence of collagen protein. Our qualitative amino acid analysis shows the presence of high concentrations of hydroxproline and glycine which is characteristic of both vertebrate and invertebrate collagen (Astbury, 1947; Watson and Smith, 1956; Watson, 1958). We believe the fine fibrils seen in the orthogonal tubules are actually collagen fibrils. The term "tubules" is used only as a descriptive term. This is the only example of collagen tubules we know. Our observations made on cuticle of *Nereis limnicoli* and *Stauronereis rudolphi* show solid fibers like those reported for *Syllis* (Boilly, 1970). The orthogonal arrangement of collagen fibers is common and has been described in cuticle of nemerteans, molluscs, echinoderms and the basement lamella of platyhelminthes, fish and amphibians as well as human

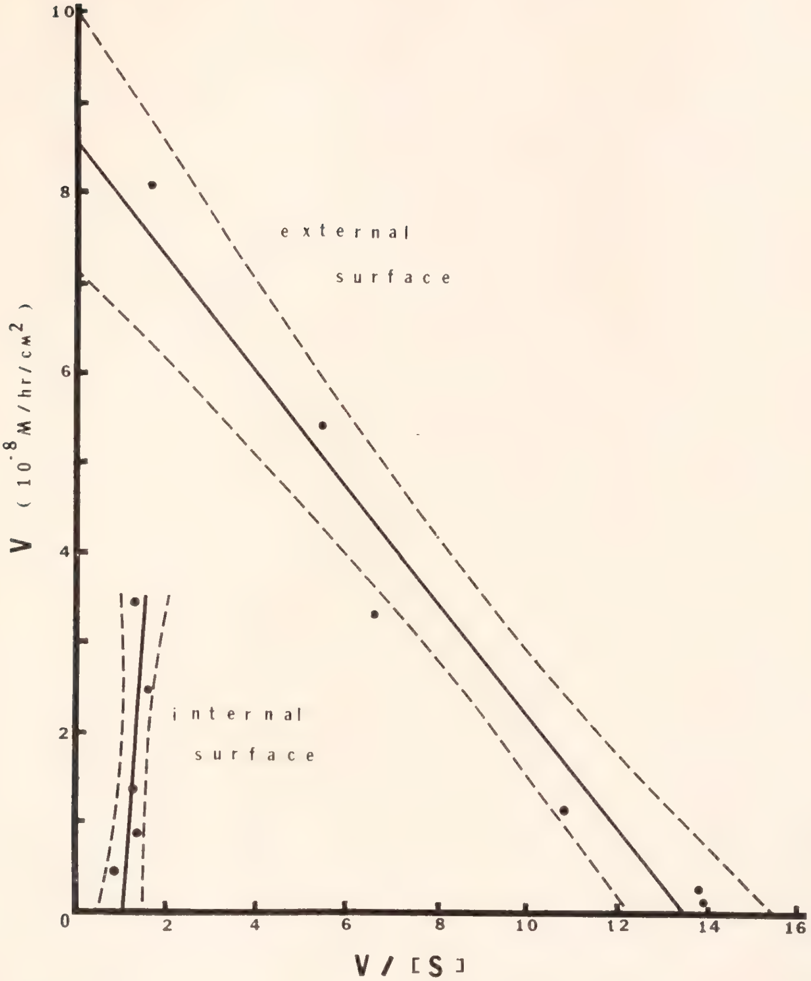

FIGURE 9. Hofstee's linear transformation of the Michaelis-Menten equation for taurine uptake across the external and internal surface of isolated pieces of body wall. The broken lines indicate the 95% confidence intervals. The lines were fitted by least squares method.

cornea (see references in Nadol, Gibbins and Porter, 1969; Lloyd, 1969; Menton and Eisen, 1970).

We found neither bacteria nor fungi associated with the external surface of *Glycera* in any scanning or transmission electron micrographs. However, small dense epicuticular particles were routinely present. Similar particles have been observed in all oligochaetes studied (Potswald, 1971). Several hypotheses concerning their nature and origin have been advanced (Reed and Rudall, 1948; Coggeshall, 1966). It seems clear now that they are derived from the tips of the epidermal microvilli (Hess and Menzel, 1967; Potswald, 1971). However, the function of these particles is virtually unknown. We merely report their presence in *Glycera.*

The presence of epidermal microvilli is very common in soft bodied invertebrates. Recent reports of microvilli in other groups include the platyhelminthes (MacRae, 1967), the molluscs (Lloyd, 1969), the pogonophorans (Little and Gupta, 1969) and the echinoderms (Menton and Eisen, 1970). Their presence is often accompanied by a cuticle or mucilagenous material or both. Little is known about the function of these microvilli. Many speculations have been made. Since they are in direct contact with the cuticle or mucus, a role in secretion and maintenance of these extracellular materials has been suggested. (Lane, 1963; Coggeshall, 1966; Potswald, 1971). Potswald (1971) observed the temporary elaboration of numerous microvilli during regeneration of the cuticle of an oligochaete. We wish to focus attention on the possible role of microvilli in absorption of material from the environment. Uptake of small organic compounds by free-living marine invertebrates has been well documented (Stephens, 1968). The resemblance in ultrastructure between the epidermal microvilli of invertebrates and the vertebrate intestinal brush border has been pointed out by many authors (Lane, 1963; Nørrevang, 1965; Little and Gupta, 1969; Potswald, 1971).

We have demonstrated that whole *Glycera* worms accumulate neutral, acidic and basic amino acids. The K_t's for eight different amino acids vary from 2×10^{-4} to 2×10^{-3} moles/liter. These values fall in the upper range of reported K_t values (5×10^{-5} to 2×10^{-3}) for most marine invertebrates. Stephens (in press) and Southward and Southward (1970) review the evidence and conclude that K_t values for marine organisms are related to the free amino acid concentrations in their normal habitats. This generalization applies to *Glycera* which live in muddy sediments (Klawe and Dickie, 1957) where dissolved organic substances are likely to be abundant.

Passage of amino acids into the body wall occurs more rapidly across the epidermis than the peritoneum. The microvilli on the external surface of *Glycera* provide a surface area about seven times greater than that of the internal surface (13.8: 1.8). This ratio roughly agrees with the ratio of influx rates determined by the Ussing chamber experiments. Probably the agreement is accidental since examination of Figure 8 shows that the ratio would depend on the concentration of amino acids present in the medium.

Further characterization of these two surfaces by the kinetic studies shows that the influx across the internal surface of *Glycera* body wall is directly proportional to the medium concentration, over a wide range, suggesting entry by passive diffusion. The apparent passive behavior of the peritoneum regarding amino acid influx is interesting in connection with the distribution and maintenance of intracellular amino acid pools. Preston and Stephens (1969) and Preston (1970) have shown that the high amino acid concentrations in *Glycera* coelomocytes are due to the ability of these circulating cells to accumulate such compounds. The interaction of passive and facilitated distribution of amino acids between the coelomic cells, coelomic fluid and the body wall remains to be described in detail.

In contrast to the internal surface, the external surface shows saturable kinetics which characterize other mediated transport systems. The K_t for taurine agrees well with the K_t values for other amino acids determined for the whole worms

(Table II). Therefore, the ultrastructural and uptake studies lead to the conclusion that differentiation to increase the surface area and the intrinsic properties of the cell membrane are both important in the transport of dissolved organic molecules across the epidermis of *Glycera*.

We would like to express our thanks to Dr. A. Loeblich II for the use of the JEOL microscope; Mr. R. MacAdam and Miss J. Kiethe for technical assistance in scanning electron microscopy; Mr. J. Biela for help in construction of an experimental chamber.

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SUMMARY

1. The epidermis of *Glycera* consists of interdigitating columnar cells and mucus secreting goblet cells. Both types of cells are covered by a collagenous cuticle, 1 to 12 μm thick.

2. The cuticle is made up of layers of orthogonally arranged tubules in an amorphous matrix. Individual tubules measures 0.1 to 0.3 μm in diameter and are composed of collagen fibers about 5 nm thick.

3. Microvilli extending from the columnar epithelial cells into the cuticle increase the surface area of these cells about 13 times.

4. The peritoneum which covers the coelomic surface of the body wall is composed of one to several layers of flattened cells. Irregular cytoplasmic projections double the surface area.

5. *Glycera* whole animals take up eight different amino acids by saturable systems. The K_t values range from 2×10^{-4} to 2×10^{-3} moles/liter, which fall in the upper range of reported K_t values for marine invertebrates.

6. Influx of amino acids occurs across both surfaces of isolated pieces of body wall. The kinetic characteristics of transport across the epidermis are similar to those of the whole worms.

7. Both the ultrastructure and the physiological differentiation of the epithelial cells are shown to be important in the transport of amino acids across *Glycera* body wall.

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BENEFIT TO SYMBIOTIC ZOOCHLORELLAE FROM FEEDING BY GREEN HYDRA [1]

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The study of various algal-invertebrate symbioses has largely concerned the effects of endosymbiotic algae on their invertebrate partners. The most conclusive of these studies have shown that the green algae (zoochlorellae) symbiotic with fresh-water invertebrates augment the survival of the host during periods of low food supply (Pringsheim, 1928; Karakashian, 1963; Muscatine and Lenhoff, 1965). This benefit is probably due to low molecular weight compounds of photosynthetic origin which the algae pass on to their hosts (Muscatine and Lenhoff, 1963; Muscatine, Karakashian and Karakashian, 1967).

The possibility that the algae also benefit from these associations is suggested by several reports in the literature. Yonge and Nicholls (1931) concluded that dinoflagellates (zooxanthellae) symbiotic with scleractinian corals removed waste products from coral tissue; presumably the algae could utilize these products in their own metabolism. Siegel (1960) reported that zoochlorellae survived in *Paramecium bursaria* after 100 generations of growth of the host in darkness. One implication of this finding is that the algae utilized heterotrophic carbon for growth in darkness, and that bacteria fed to the paramecia may have been the source of this carbon. Finally, uptake of ^{35}S by symbiotic algae from food ingested by animal hosts is indicated in green hydra (Muscatine and Lenhoff, 1965) and in a sea anemone (Cook, 1971).

The benefit implied by these examples is that food ingested by animal hosts is a nutrient source for algal endosymbionts, although rigorous experimental proof of this is lacking. The present paper presents evidence that feeding by green hydra promotes the growth of its symbiotic zoochlorellae under conditions which necessitate nutrient flow from the hydra to the algae.

GENERAL MATERIALS AND METHODS

Green hydra were purchased from Carolina Biological Supply Co. (Burlington, North Carolina). The company identifies these animals as *Chlorohydra viridissima,* although the taxonomic status of these organisms appears to be in doubt (Oschman, 1967). All hydra used in my experiments were derived from these stocks. Stocks for all experiments were grown in M-solution (Muscatine, 1961) using the techniques of Loomis and Lenhoff (1956). All stock cultures were maintained at 22 ± 1° C under 24-hour fluorescent illumination (2000 lumens/m^2 at the surface of the cultures). In all experiments, "uniform hydra" were selected from stock cultures which had been fed brine shrimp nauplii daily for at least fourteen days prior to use. "Uniform hydra" are defined by Lenhoff and Bovaird (1961) as being hydra from logarithmically growing cultures which have one small bud and which have been starved for one day. These authors discuss why the animals may be considered uniform in size and in distribution of cellular components.

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[1] This paper is based on a portion of a dissertation submitted to the Graduate School of Arts and Sciences of Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology.

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Results

Effect of feeding on growth of algal symbionts in light

Triplicate samples of five "uniform" hydra taken from constant light stocks were fed an excess of freshly hatched brine shrimp nauplii at intervals ranging from once daily to once every four days for thirteen days; illumination and temperature conditions were as described for the maintenance of stock cultures. All culture solutions were changed one hour after feeding and again five hours later; during the second change the hydra were removed from their dishes and the dishes wiped clear of accumulated debris. The hydra were returned to their dishes with clean culture solution.

On the fourteenth day after the start of the experiment, the hydra in each dish were counted and then homogenized with a tissue grinder. I separated algal cells from the suspension by centrifugation (275 *g* for ten minutes). After three washes with deionized water the algae were collected on membrane filters (0.45 μ^2 mesh); these filters were mounted on microscope slides and the algae counted with the aid of a phase contrast microscope.

The final number of algal cells in each culture fed in the light increased with increased feeding frequency (Fig. 1, white histograms). The number of algal cells per hydranth appeared to remain constant (Fig. 2, white histograms).

These results imply that the growth rates of green hydra and its symbiotic algae are similar. Since the doubling time of these hydra when fed daily in constant light is 2.0 days (Cook, 1970), this presumably is the doubling time of the algae grown under these conditions. This is a slower growth rate than that of autotrophic *Chlorella* strains in culture (Samajima and Myers, 1958) and may indicate some "limitation" of the growth of the symbionts. It is also possible that "excess" algae leave the hydra. Taylor (1969) reports that the endosymbiotic zooxanthellae of a sea anemone are occasionally released from the animal; I made no attempts to quantify any such losses of algal cells to the medium.

Effect of feeding on growth of algal symbionts in darkness

To quantify possible heterotrophic growth of the algae the above experiment was repeated in darkness. Triplicate samples of five "uniform" hydra taken from stocks grown under constant illumination were fed and cleaned as described in the previous section. These experimental cultures were maintained in a closed box painted black on the inside; the boxes were kept in a light-tight drawer. Feeding and cleaning of these cultures occurred under safelight illumination (Kodak Wratten OA, 20-watt bulb at twenty feet). Total safelight exposure did not ex-

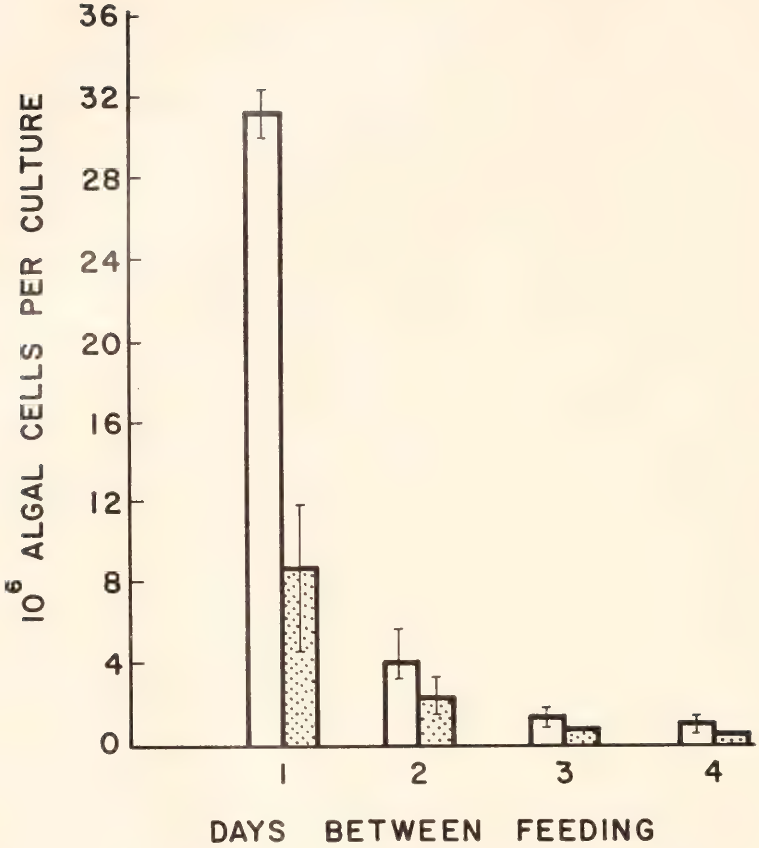

FIGURE 1. The algal cell content of hydra cultures after 13 days of feeding at various frequencies. The data represent the mean number of algal cells in each culture dish at the termination of the experiment. The white histograms represent the algal cells from cultures fed in constant light; the stippled histograms represent the number of algal cells from hydra fed in darkness. The vertical bars represent the range; N = 3 for all samples.

ceed one hour per day. Counter tops and solution changes were examined for animals which may have been poured off; these animals were returned to the proper dish. After fourteen days the hydra and the algal cells were counted as described for the light experiment.

In darkness as well as in light increased feeding of the hydra resulted in increased algal growth (Fig. 1, stippled histograms), although the absolute number of algal cells at any feeding frequency in the dark was always less than that of cultures fed in the light at the same rate. Comparisons of growth rates of hydra and algae in darkness (Fig. 2, stippled histograms) imply that, as in the light, bud production by the hydra and increase in algal cell numbers occurred at similar rates.

It follows from the above results that growth rates of hydra in darkness were lower than growth rates of hydra fed at corresponding rates in the light. This lower growth rate in darkness is also suggested by the data of Stiven (1965),

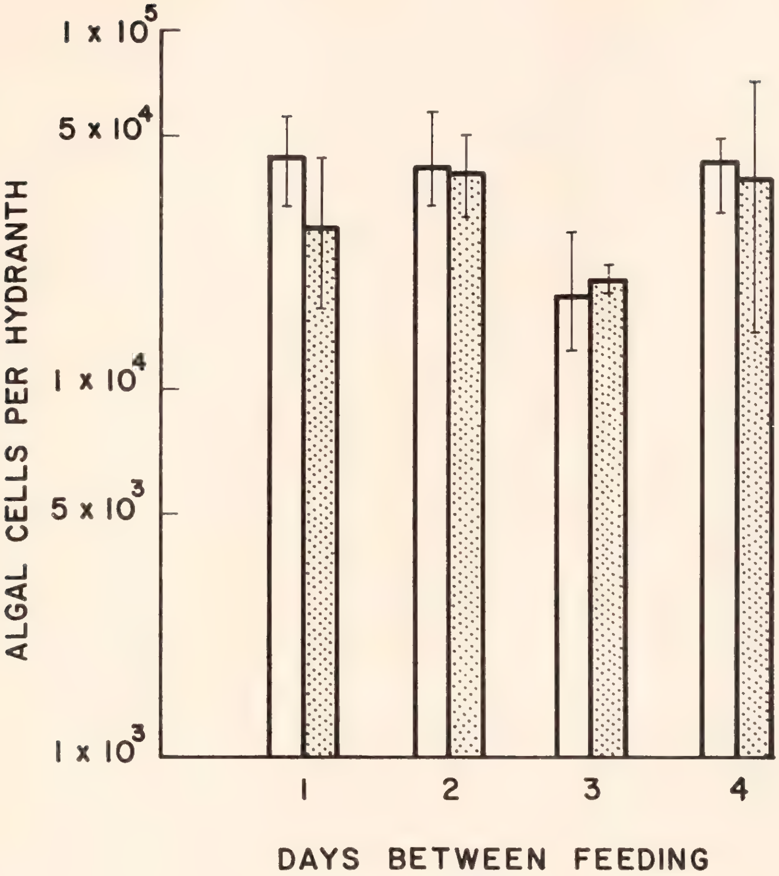

FIGURE 2. The number of algal cells per hydranth in hydra cultures after 13 days of feeding at various frequencies. A hydranth is defined as any bud. The white histograms represent cultures fed in continuous light; stippled histograms represent cultures fed in darkness. The vertical bars represent the range; N = 3 for all samples.

particularly when hydra were fed less often than once every 24 hours. However, in my studies I cannot exclude the possibility that hydra were lost during the feeding and cleaning periods; such losses would imply that the growth rates of both hydra and algae in darkness were greater than I found.

Carbon fixation by green hydra in light and darkness

The results of the previous section suggested that the algae growing in darkness utilized heterotrophic carbon sources for growth; however, it is possible that photosynthesis may have occurred in the dark cultures, particularly during the periods of feeding and cleaning when the animals were exposed to the safelight. To assay the relative amounts of photosynthesis in the constant light and dark conditions I exposed "uniform" green hydra in "M-solution" containing $NaH^{14}CO_3$

(0.27 μc/mg, 0.25 μc/ml) to either 24 hours of constant light or to one hour of safelight followed by 23 hours of darkness. Radioactivity of the hydra was assayed by the method of Muscatine and Lenhoff (1963).

Hydra in the dark fixed less than 1½% of the carbon fixed by hydra in the light. Since hydra tissue is capable of heterotrophic CO_2 fixation (Lenhoff, 1959), I take this result to indicate that photosynthesis by the dark cultures was negligible compared to that in the light so that the algae may be using exogenous reduced carbon sources for growth in darkness.

Uptake of ^{14}C by algae from food ingested by green hydra

One source of the reduced carbon used by the algae for growth in darkness could be food ingested by the hydra. I investigated the possible uptake by the algae of carbon from ingested food in preliminary studies by feeding ^{14}C-labelled brine shrimp nauplii to green hydra. Yeast cells were labelled with D-glucose-^{14}C (uniformly labelled; New England Nuclear) and were fed to freshly hatched brine shrimp nauplii; the larvae became radioactive within 2 days. The labelled nauplii were then fed to hydra in both continuous light and continuous darkness. Forty-eight hours after feeding, pooled samples of 5–10 hydra were homogenized, and the algae separated by centrifugation (270 *g*). Washed algal pellets were collected on Millipore filters (0.45 μ^2 mesh) and the filters glued onto planchets. Aliquots of algae-free hydra supernatants were also placed on planchets.

Analysis of the distribution of label between hydra supernatants and algal pellets in two trials in the light showed that 22% and 26% of the total recovered radioactivity was in the algal fraction, while two samples kept in continuous darkness yielded 25% and 34% of the total radioactivity in the algal fraction. Estimates of contamination showed that no more than 10% of the radioactive hydra tissue is recovered in the algal fraction by this technique (Cook, 1970); thus it appears that carbon from ingested food is taken up by zoochlorellae in both light and darkness.

DISCUSSION

"M-solution" contains no phosphorus, sulfur, or trace elements, and no nitrogen except that found in "Tris" buffer (Muscatine, 1961); therefore these inorganic nutrients must be supplied to both hydra and algae by the food of the hydra in both light and darkness. This is borne out in tracer experiments in which ^{35}S has been traced from ingested food to the zoochlorellae of green hydra cultured in "M-solution" (Muscatine and Lenhoff, 1965). Since photosynthesis by green hydra in the dark experiments described in this paper is negligible, and since ^{14}C is taken up by the algae from ingested food, it follows that the food of the hydra is the source of some of the reduced carbon used by the algae for growth in darkness. It is also possible that the algae use the "Tris" present in "M-solution" as a source of organic carbon for this purpose.

Three lines of evidence suggest that organic nutrients may also flow from coelenterates to algal endosymbionts in the light, and that these nutrients are metabolically used by the algae. First, zoochlorellae take up ^{14}C from food ingested by hydra in the light (this paper), although this may represent photosynthetic incorporation of CO_2 produced by the hydra; the resolution of this question will depend on the analysis of labelled compounds in the algal and animal fractions. Secondly, several independent reports indicate that the symbionts of green hydra have not been grown successfully in culture outside the hydra (Loefer, 1936; Park, Greenblatt, Mattern and Merril, 1965; Muscatine *et al.*, 1967). This is not the case for zoochlorellae isolated from *Paramecium bursaria* (Loefer, 1936) and *Spongilla lacustris* (Muscatine *et al.*, 1967), and perhaps indicates the loss of metabolic pathways in the hydra symbionts. Presumably these pathways would have normally synthesized metabolites which can be supplied by the hydra so that the algae have become dependent upon the hydra to supply certain "essential" nutrients.

Finally, the possibility of a metabolic loss in algal endosymbionts is indicated by the work of von Holt (1968). He found that the dinoflagellates (zooxanthellae) symbiotic with a West Indian zoanthid contained no free glycine labelled with ^{14}C after a 3 hour incubation with $^{14}CO_2$, even though labelled glycine was detectable in animal tissue. This result suggests that the algae are unable to synthesize glycine from photosynthetic precursors, at least within the 3-hour limit of the experiment, so that the algae may have to depend upon the animal to supply this amino acid.

The selective value of such metabolic "defects" has been experimentally demonstrated in bacteria (Zamenhof and Eichhorn, 1967). These workers found that "defective" bacteria grew faster than "wild-type" bacteria when both are grown on complete media and suggest that such defective bacteria may benefit because they do not have to manufacture the DNA and synthetic machinery required for deleted pathways. It is conceivable that a similar situation may exist in the green hydra symbionts; metabolic blocks in carbon metabolism should therefore be looked for in these algae.

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SUMMARY

1. The symbiotic zoochlorellae of green hydra grow faster in both light and darkness with increased feeding by the hydra, although growth of algae and of hydra fed at similar frequencies is always less in darkness.

2. The algal cell content of green hydra is similar under all conditions of feeding in both light and darkness.

3. Photosynthesis by green hydra in darkness is negligible when compared to that by hydra in the light.

4. The algae take up ^{14}C from brine shrimp ingested by the hydra in both light and darkness.

5. It is suggested that food ingested by the hydra serves as a source of inorganic nutrients for the algae, as well as a pool of organic nutrients which the algae utilize for heterotrophic growth in darkness and which the algae may require for growth in the light.

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ENDOCRINE INFLUENCES ON SEMINAL VESICLES IN THE ESTUARINE GOBIID FISH, *GILLICHTHYS MIRABILIS*

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Seminal vesicles, which occur as paired glandular structures attached to the posterior region of the common spermatic duct in male teleost fishes, were noted by Rathke (1824) in the goby, *Gobius niger*. Since then, seminal vesicles have been observed and briefly described in several other gobiid fishes (Hyrtl, 1850; Eggert, 1931; Weisel, 1949; Tavolga, 1955; Egami, 1960) as well as in many other fishes (Hyrtl, 1850; Disselhorst, 1904; Gudger, 1908; Ihering, 1937; Gilbert, 1943; Sundararaj, 1958; Nawar, 1959; Hoffman, 1963; Sircar, 1966, Lehri, 1967; Rastogi, 1969; Nayyar and Sundararaj, 1970a). The seminal vesicles of *Gobius paganellus* (Vivien, 1938), *Heteropneustes fossilis* (Sundararaj, 1958), *Clarias lazera* (Nawar, 1959), *Opsanus tau* (Hoffman, 1963), and *Mystus tengara* (Rastogi, 1969) exhibit seasonal development and regression closely following changes in the testes. On the other hand, Weisel (1949) has reported that in *Gillichthys mirabilis*, an estuarine gobiid fish, the seminal vesicles do not show cyclicity; the amount of secretion and the appearance of the secretory epithelium do not change appreciably in the various seasons.

The literature on the endocrine regulation of teleost sex accessories was reviewed by Pickford and Atz (1957), Dodd (1955, 1960), Hoar (1957, 1969) and Bern and Nandi (1964). However, data on endocrine influences on the secondary sex accessories such as the seminal vesicles are meager. The seminal vesicles of teleosts are not homologous with their mammalian counterparts inasmuch as they do not arise from the Wolffian duct. There is no unanimity of opinion with regard to the response of the seminal vesicles to androgen. Weisel (1949) indicated that the seminal vesicles of *Gillichthys mirabilis* do not respond to testosterone. Sundararaj and Goswami (1965a, 1965b), Sundararaj and Nayyar (1967, 1969a, 1969b, 1969c) and Nayyar and Sundararaj (1969, 1970b) have presented a detailed picture of endocrine control of the seminal vesicles in the catfish, *Heteropneustes fossilis*.

The seminal vesicles of *Gillichthys mirabilis* do not show cyclical changes, whereas the testes have a distinct annual cycle (de Vlaming, 1972). This indicates that the secretory activity in the seminal vesicles may be regulated by a direct pituitary principle in addition to possible testicular influences. Ths investigation on the estuarine gobiid fish, *Gillichthys mirabilis*, was undertaken (1) to study the effects of castration or hypophysectomy on the secretory seminal vesicles and, (2) to determine the response of the seminal vesicles of the hypophysectomized specimens to administration of testosterone propionate and ovine prolactin, singly and in combination.

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MATERIALS AND METHODS

Specimens of *G. mirabilis* were collected in January from the Alviso salt ponds at the southern end of San Francisco Bay in California; this habitat was previously described and the reproductive cycle of this population of *G. mirabilis* educidated (de Vlaming, 1971a, 1972).

Due to the large gape of *Gillichthys*, hypophysectomy can be performed by entering through the mouth. The hypophysis of this fish is situated just posterior to the first gill cleft in the cranial floor. After removal of a small section of the buccal epithelium, the gland is readily visible as a white oval body. Fish were anesthetized with 1:4000 tricaine methane sulfonate (MS 222) dissolved in sea water. Animals were then placed belly upward in a groove made in a large cork. The lower jaw was held open with a hook attached to a ringstand. After cutting away a section of the buccal epithelium, a small hole was made in the parasphenoid bone just anterior to the pituitary with a (No. 5) dental drill. Two slits were then cut posteriorly from this hole and the bone folded back. The pituitary was removed by vacuum. The bone was then folded back into place and covered with buccal epithelium. Fish were returned to sea water following surgery. For further details on hypophysectomy of *Gillichthys* see de Vlaming (1971b).

Fish to be castrated were anesthetized with MS 222. A short incision was made to the left of the mid-ventral plane in the lower abdomen. Due to the loose skin of *Gillichthys* this incision could be moved so that each of the testes could be observed. The testes could be easily removed through the incision by grasping them firmly with forceps. The sperm duct leading from each testis was cut approximately 3 mm anterior to the junction with the seminal vesicle. The incision was carefully sutured with surgical silk and the area cleaned with 70% ethanol. This procedure has been thoroughly discussed by Sundararaj and Goswami (1965b).

Hormone treatments were begun seven days after hypophysectomy. Each fish received a total of seven daily injections and were sacrificed the day after the last injection. Castrate fish were sacrificed 14 days after castration. Testosterone propionate (Mann Research Laboratories) was injected in 1% solution of Tween and ovine prolactin (NIH-P-S8) in a 0.6% saline solution. The volume injected was kept constant at 0.1 ml. All experimental fish weighed between 35 and 50 g. The experimental groups and hormone dosages are presented in Table I.

Experimental fish were maintained in 56 liter fiber glass tanks. These tanks were provided with recirculating filtered sea water. Animals were fed every other day on chopped beef liver. Further details on maintenance of *Gillichthys* can be obtained in de Vlaming (1971b, 1971c). Water temperature was regulated at 18° ± 1° C since this temperature promotes reproductive activity (de Vlaming, in preparation) and is within the preferred temperature range of this species (de Vlaming, 1971a). The lighting schedule was 13 hr of light and 11 hr of dark.

Upon termination of the experiments, fish were killed with a saturated solution of chlorotone and weighed. The seminal vesicles were dissected out, weighed and

TABLE I

Effect of testosterone propionate (TP) and prolactin (LtH) on the seminal vesicles of hypophysectomized Gillichthys mirabilis

Group No.	n	Treatment for 7 days, daily dosage		Seminal vesicle Wt. (mg)/Body wt. (g) ($\bar{x}$ ± S.E.)*	Area of 8 largest vesicular lumina (mm^2) ($\bar{x}$ ± S.E.)**
		TP (μg)	LtH (μg)		
Sham operated					
I	5	0	0	2.84 ± 0.28	47.53 ± 4.16
Hypophysectomized					
1	6	0	0	0.38 ± 0.08	16.15 ± 4.00
2	6	25	0	0.11 ± 0.02	1.67 ± 0.67
3	6	150	0	1.49 ± 0.35	35.06 ± 4.87
4	6	0	200	0.96 ± 0.21	44.21 ± 7.02
5	6	25	200	4.00 ± 0.96	60.12 ± 7.59

* P values (Mann-Whitney U test): I and 1: $P < 0.001$; I and 2: $P < 0.001$; I and 3: $P < 0.01$; I and 4: $P < 0.001$; 1 and 2: $P < 0.05$; 1 and 3: $P < 0.01$; 1 and 4: $P < 0.05$; 1 and 5: $P < 0.001$; 2 and 3: $P < 0.01$; 2 and 4: $P < 0.01$; 2 and 5: $P < 0.001$; 3 and 5: $P < 0.05$; 4 and 5: $P < 0.01$.

** P values: I and 1: $P < 0.001$; I and 2: $P < 0.001$; I and 3: $P < 0.05$; 1 and 2: $P < 0.001$; 1 and 3: $P < 0.01$; 1 and 4: $P < 0.001$; 1 and 5: $P < 0.001$; 2 and 3: $P < 0.001$; 2 and 4: $P < 0.001$; 2 and 5: $P < 0.001$; 3 and 5: $P < 0.05$; 4 and 5: $P < 0.05$.

weights were standardized by dividing them by the body weight of the fish. The seminal vesicles fixed in alcoholic Bouin's fluid and embedded in paraffin for histological examination. Sections were cut 8–9 μ and stained with Harris' hematoxylin and eosin. Camera-lucida drawings were made of the eight largest lobules of the seminal vesicles of each fish. The area of the lumina of these lobules was determined using a planimeter. Mean area was then determined for each experimental group (see Table I).

Statistical comparisons between experimental groups were made by using the Mann-Whitney U test (Siegel, 1956). This nonparametric test is suitable for small sample sizes and can be used to determine whether two independent groups have been drawn from the same population.

RESULTS

Weisel (1949) has described the seminal vesicles of *G. mirabilis*. In the sham-operated fish, the seminal vesicles were enlarged and distended with secretion. The secretory epithelium was hypertrophied (Table I).

Fourteen days after castration, the seminal vesicles of four of the five castrate fish were regressed so completely that they could not be easily dissected out. The weight (mg seminal vesicles/g body weight) of the seminal vesicles of the remaining castrate fish was 0.43. No colloid remained in the lobules of the seminal vesicles of this fish and the secretory epithelium was degenerated.

Hypophysectomy also caused a regression of the seminal vesicles. The weight of the seminal vesicles and area of the vesicular lumina in the 14-day hypophysectomized fish were significantly less ($P < 0.001$) than in the sham-operated controls (Table I). Very little colloid remained in the lobules of the hypophysectomized fish and the secretory epithelium was beginning to degenerate.

The effects of the various hormone treatments are summarized in Table I. A low dose (25 μg/day) of testosterone propionate (TP) did not inhibit seminal vesicle regression in the hypophysectomized fish (Table I). Rather, it accentuated regression. On the other hand, a higher dose (150 μg/day) of TP did prevent regression of the seminal vesicles in the hypophysectomized specimens. This treatment maintained the secretory epithelium and colloid was present in the lobules. The seminal vesicle weight and area of the lumina of the lobules in the TP-treated (150 μg/day) group were significantly greater ($P < 0.01$) than in the hypophysectomized control fish. Seminal vesicle activity in this group, however, was slightly less than in the sham-operated controls (Table I).

Treatment with mammalian prolactin also prevented regression of the seminal vesicles in the hypophysectomized specimens. The vesicular epithelium was well-maintained and colloid filled the lumina. Nonetheless, the seminal vesicle weights in the prolactin-treated group were significantly less ($P < 0.001$) than in the sham-operated group, but the areas of the lobule lumina were not significantly different in these two groups. Compared to the hypophysectomized controls, seminal vesicle weights ($P < 0.05$) and luminal areas ($P < 0.001$) were significantly greater in the prolactin-treated group. The seminal vesicles of the prolactin-treated fish and those of fish receiving a high dose (150 μg/day) of TP appeared to be in a similar condition.

Treatment with a combination of prolactin and a low dose of TP (25 μg/day) was effective in maintaining seminal vesicle activity at a level comparable to that in the sham-operated controls (Table I). The seminal vesicle weights and luminal area were significantly greater in this group than in those receiving either TP or prolactin alone (see Table I for P values).

DISCUSSION

In the gobiid fish, *G. mirabilis*, castration or hypophysectomy causes regression of the seminal vesicles. Regression of similar structures following hypophysectomy was also observed in several other fishes (Vivien, 1938, 1941; Tavolga, 1955; Sundararaj and Goswami, 1965b; Sundararaj and Nayyar, 1969a, 1969b). Our data thus suggest that the seminal vesicles in *G. mirabilis* are androgen dependent; hypophysectomy may cause regression by reducing or inhibiting androgen production by the testes. In contrast to our results with *G. mirabilis*, however, Sundararaj and Goswami (1965b), Sundararaj and Nayyar (1969b, 1969c) and Nayyar and Sundararaj (1969) noted that castration during the prespawning period stimulated hypersecretory activity in the seminal vesicles of the catfish, *Heteropneustes fossilis*. These workers found that the high titers of gonadotropin following castration stimulated the interrenal to produce androgens which in turn acted on the seminal vesicles to induce hypersecretion (Nayyar and Sundararaj, 1969; Sundararaj and Nayyar, 1969b, 1969c).

In *G. mirabilis* treatment with testosterone propionate (TP) or ovine prolactin prevents regression of the seminal vesicles in the hypophysectomized fish. But neither of them alone was effective in maintaining the seminal vesicles at the level of activity noticed in the sham-operated controls. Sage and de Vlaming (unpublished data) have also noted that prolactin inhibits regression of the seminal vesicles in the hypophysectomized *G. mirabilis.* On the other hand, Weisel (1949) reported that the seminal vesicles of *G. mirabilis* do not respond to testosterone; his experiments, however, were with intact fish injected with testosterone only three times at 7-day intervals or suspended in the aquarium water. More experimental data are needed to determine whether or not both hormones promote growth and secretion in the completely regressed seminal vesicles. Sundararaj and Goswami (1965b) and Sundararaj and Nayyar (1969a) have reported that in the hypophysectomized catfish, *Heteropneustes fossilis,* prolactin alone does not initiate secretory activity in the seminal vesicles, whereas androgen alone is effective (Sundararaj and Goswami, 1965a; Nayyar and Sundararaj, 1970b).

Only a combination of prolactin and TP was effective in maintaining the seminal vesicles at the level of activity seen in the sham-operated controls. Sundararaj and Goswami (1965b) and Sundararaj and Nayyar (1969a) have shown that prolactin promotes growth and secretion in the seminal vesicles of the catfish, *Heteropneustes fossilis,* only in the androgen-primed fish; thus, their data suggest a synergism between prolactin and testosterone—neither hormone being as effective as a combination of the two. A similar synergism is apparently operative in *G. mirabilis.*

Prolactin in synergism with low levels of androgen (which alone do not stimulate seminal vesicles) is very effective in maintaining the secretory seminal vesicles of the hypophysectomized *G. mirabilis.* In the fish treated wth prolactin alone, the possibility of low titers of androgen being released from the testes or interrenal cannot be ruled out. The above suggestion at least in part may explain the continued activity of the seminal vesicles even at a time when the testes are regressed. Since prolactin is capable of synergizing with very low levels of androgen, seminal vesicles may presumably be activated or at least maintained whenever prolactin is released. In *G. mirabilis* prolactin brings about dispersal of pigment in the xanthophores (Sage, 1970). Further, the fish when placed in fresh water turns yellow—an indication of prolactin release, although there is no evidence that prolactin has an osmoregulatory role in this species (Sage, 1970). Even so, it is not unreasonable to expect the release of prolactin in *G. mirabilis* with decrease in the environmental salinity. *G. mirabilis* is afterall an estuarine fish capable of tolerating wide fluctuations in salinity. If prolactn is at all involved in osmoregulation, its relase brought about by changes in salinity, would also affect the seminal vesicles even though the testes may be regressed, provided androgen is present. Another possibility is that prolactin stimulates androgen production by either the testes or interrenal. Although this suggestion seems unlikely, Ball and Fleming (unpublished data cited in Ball and Ensor, 1969) and Chambolle (1967) have shown that prolactin does stimulate the interrenal in some teleosts.

The lack of any obvious seasonal cycle in the seminal vesicles of *G. mirabilis* indicates that throughout the year the titers of prolactin and/or androgen are sufficient to maintain some activity. Although there is a clear-cut cyclicity in the testes of this fish (de Vlaming, 1972), androgen production by the testes or interrenal may remain at a level sufficient to synergize with prolactin in maintaining seminal vesicle activity. Further experiments are necessary to examine this possibility.

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SUMMARY

1. The effects of castration or hypophysectomy on the seminal vesicles of the gobiid fish, *Gillichthys mirabilis* were examined. In addition, the influence of ovine prolactin and testosterone propionate treatments was determined in the 7-day hypophysectomized specimens.

2. Both castration and hypophysectomy caused regression of the seminal vesicles,

3. A low dose of testosterone propionate (25 μg/day) did not inhibit regression of the seminal vesicles in the hypophysectomized fish.

4. A high dose of testosterone propionate (150 μg/day) or prolactin treatment prevented regression of the seminal vesicles in hypophysectomized fish; neither treatment, however, maintained the level of activity observed in sham-operated fish.

5. A combination of testosterone propionate and prolactin maintained seminal vesicle activity in the hypophysectomized fish at a level equal to that in sham-operated controls.

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THE DEVELOPMENT OF THE OVOTESTIS AND COPULATORY ORGANS IN A POPULATION OF PROTANDRIC SHRIMP, *PANDALUS PLATYCEROS* BRANDT FROM LOPEZ SOUND, WASHINGTON [1]

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The large commercially important shrimps of the decapod family Pandalidae demonstrate high incidences of protandric hermaphroditism (Berkeley, 1929, 1930). Almost all of the individuals pass through a functional male stage of one to three years before undergoing transformation to a functional female stage. During the male stage the gonad is an ovotestis, but only the testicular elements are functional. Whereas, in the female stage the gonad is a true ovary, the testes having degenerated during sexual transformation.

The incidence of protandry among the eleven different species of *Pandalus* and *Pandalopsis* from the North Pacific appears to be variable in those populations that have been studied. Some species are dominantly protandric such as *Pandalopsis dispar*, *Pandalus platyceros*, and likely *P. montagui tridens* and *P. stenolepis* (Butler, 1964). Primary females or those females that have not passed through an initial male stage have been reported in populations of *Pandalus jordani, P. hypsinotus, P. danae* and *P. goniurus* (Butler, 1964); the circumpolar *Pandalus borealis* (Allen, 1959; Carlisle, 1959a, 1959b, 1959c; Butler, 1964); *Pandalus montagui* from North Atlantic and North Sea populations (Leloup, 1936, Mistakidis, 1957; Allen, 1963). Aoto (1952) has recorded that the Japanese pandalid, *Pandalus kessleri* appears to be dominantly protandric.

The known gonochoristic species generally are found in North Atlantic populations. These include *Pandalus bonnieri* (Pike, 1952) and *Pandalus propinquus* (Jägersten, 1936). There is also evidence that certain populations of *Dichelopandalus leptocerus* are gonochoristic (Scattergood, 1952).

Although Butler (1964) and Berkeley (1930) have made intensive studies on various populations of different species of pandalids off the coast of British Columbia, there remains questions that need to be answered concerning the relations between the age classes of shrimp and the stage of gonadal differentiation. Also, further information is needed on the relationship between age and size to the phenomenon of sexual transformation.

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Materials and Methods

Pandalus platyceros Brandt is the largest of the Pacific pandalids. The females at times exceed 20 cm in length from the base of the eyestalk to the tip of the telson. This shrimp is fished heavily by commercial shrimpers throughout its range from Unalaska south to San Diego. However, the important fishing is found in the inland waters off the coast of Alaska, British Columbia, Washington and Oregon. The coloration of the shrimp is quite variable ranging from a blue-green generally found in small immature individuals to a brilliant orange. Regionally, *P. platyceros* is commonly called "The Spot" owing to a pair of white spots on the first and fifth abdominal pleura. The adults are generally found in water to a depth of 266 fathoms, but the larvae and immature forms are found in shallow inlets (Berkeley, 1930; Butler, 1964). Fishing for these shrimp is done either with the aid of trawls or shrimp pots. Butler (1964) states that the female stage is not reached until the fourth or fifth summer.

The shrimp that were utilized in this study were taken from a population that can be found in Lopez Sound, Washington, which is one of the many shallows inlets of the San Juan Archipelago. There is a depression or hole in the south central region of the sound that ranges in depth from 15 to 27 fathoms. It is in this region that abundant numbers of shrimp were found. In addition to *P. platyceros*, a large number of other species of shrimp were taken. They include, in order of relative abundance, *P. danae, P. goniurus, P. hypsinotus* and *P. stenolepis*. During the winter months, immature specimens of *Pandalopsis dispar* were also present. Although the exact number of different species was not determined, many species of hippolytid shrimp of the genera *Eualus, Spirontocaris*, and *Lebbeus* were present in this depression. In addition, a few species of *Crangon* were found.

Collection of the shrimp which generally was made on a bi-monthly basis over the period of three years (1965–1968) with the aid of a ten foot shrimp trawl.

In the laboratory, the shrimp were maintained in twenty to thirty gallon plexiglas aquaria that were supplied with running sea water. For histological studies, generally the shrimp were sacrificed within a few days of collecting. About 1,284 animals were utilized in this study.

The gonads were fixed both for wax and epoxy sections. Several different fixatives were employed for wax sections. The order of their effectiveness to fix the tissue were: Helly's, Heidenhain's "Susa," Stieve's, Zenker's and Bouin's. Helly's fixative gave the best cytoplasmic fixation whereas Heidehain's "Susa" and Stieve's fixatives were best for nuclear fixation. After initial dehydration in increasing concentrations of ethanol and tertbutyl alcohol, the tissue was enbedded in Paraplast. Sections six to seven microns were cut and mounted on albuminized slides. The stains used most frequently were the following: Gomori's Chromium Hematoxylin and Phloxine (Gomori, 1941), a modified Azan technique (Hubschmann, 1962), the Periodic Acid Schiff technique of Purves and Grieshbach (1951), and the technique employed by Halmi (1952) which was used and without the paraldehydefuchsin stain. A glutaraldehyde fixation was used for epoxy sections. Primary fixation in glutaraldehyde and postfixation in osmium is outlined in Hoffman (1969). One-half to one micron sections were strained for light microscopy using Richardson's stain (Richardson, Jarrett and Finke, 1960).

The following measurements and indices were used to determine the age and sex of the shrimp:

(1) Carapace length was used as a measurement of the size and age class. The measurement is centimeters was taken along the left side of the carapace from the base of the eyestalk to the posterior edge of the carpace (Butler, 1964). Total length proved to be too variable an index of the age of the shrimp since the lengths of the rostrum and abdomen varied in some shrimp with identical carapace lengths.

(2) The degree of development of the gonad and the sexuality of the shrimp were determined by means of histological sections. Transverse and frontal paraffin sections were made to determine general morphology and thick plastic sections of glutaraldehyde and osmium fixed tissue were made to determine the cytological aspects of the gonads.

(3) The sexuality of the shrimp was also determined by whole mounts of the copulatory organs of the first two pair of pleopods. Correlations could then be made between the secondary sex characteristics and the primary sex characters of the gonad.

RESULTS

The development of the copulatory organs

As in all natantian decapods, the male copulatory organs are located on the first two pair of pleopods. In a functional male the copulatory organ of the first pleopods consists of a swollen bulb at the distal end of the endopodite. This organ has its dorsal and lateral surface covered with hooks or cincinnuli measuring about 50 microns in length. These hooks appear to aid the shrimp in the transfer of the spermatophores to the female. The copulatory organs of the second pair of pleopods are located at the distal end of the protopodite, lateral to the endopodite. Each organ consists of two finger-like rami; the more medial is called the appendix interna and the more lateral is called the appendix masculina. The appendix interna is devoid of spines and setae except for a mass of hooks or cincinnuli along the distal lateral margin. The cincinnular mass gives the appearance of a fingernail, while the appendix interna resembles a finger. The appendix masculina of a mature male is as large as or one and a half times as large as the appendix interna. Its distal end and ventral surface are covered with broad spines. The function of these two rami in the transfer of the spermatophores is not clearly understood. During the adult development of the male phase of *Pandalus platyceros,* both of these pairs of copulatory organs undergo striking morphological changes which can be subdivided into the following stages of development (Fig. 1).

Stage 1. This stage is typical of those individuals from September through April below 2.0 cm in carapace length. The copulatory organs of the first pleopods have not yet formed (Fig. A), although the distomedial surface is free of setae and indicates the location of the future organ. The appendices internae of the second pleopods are already well formed at this stage. Their distal ends display numerous cincinnuli. However, the appendix masculinae consists of a small bud or blastema with two minute spines at the distal end. (Fig. A′).

Stage 2. Individuals between 2.0 and 2.5 cm carapace length between October and April comprise this stage. The copulatory organs of the first pleopods are

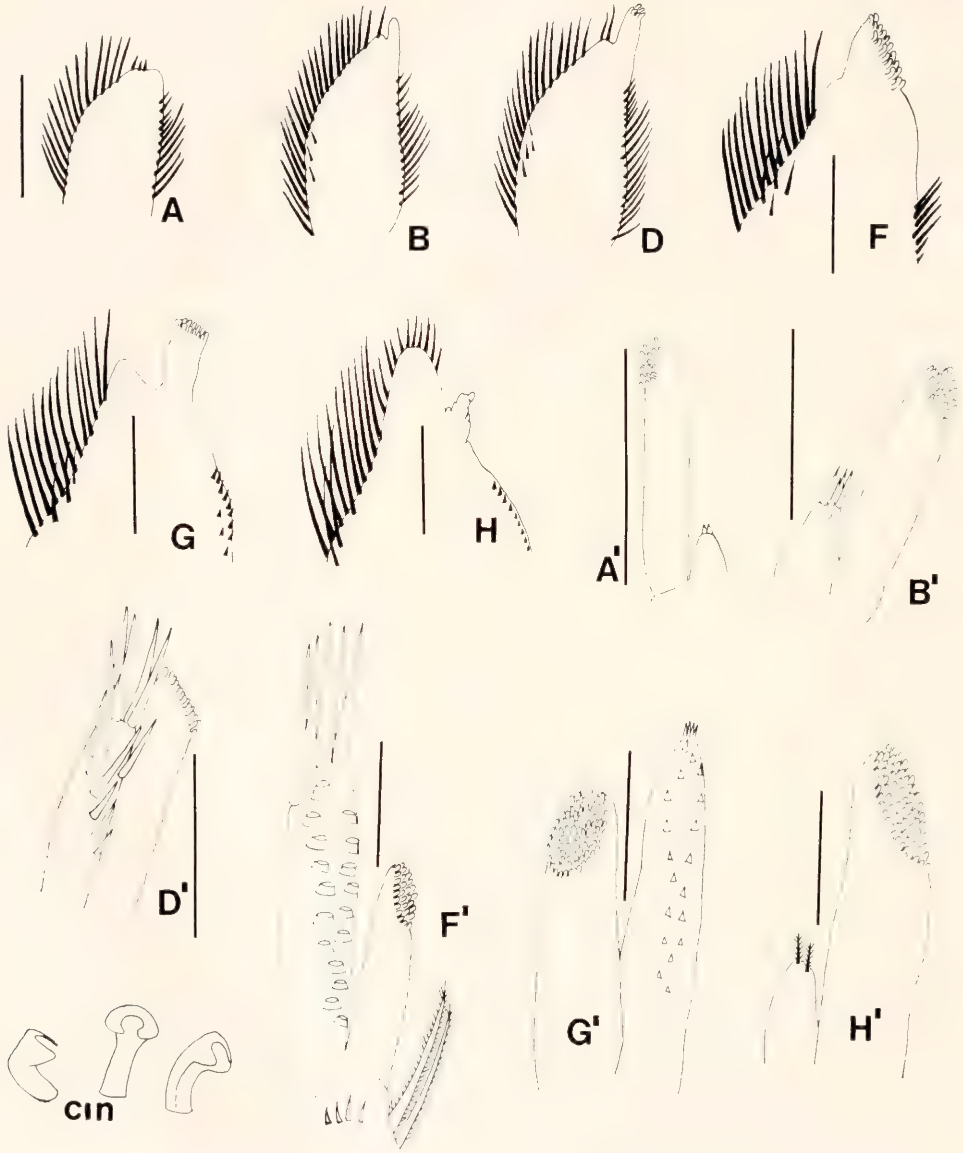


Figure 1. Copulatory appendages of male and transforming *Pandalus platyceros* Brandt; A to H, copulatory organs of endopodites of the first pleopods; A′ to H′, appendices internae and appendices masculinae of the endopodites of the second pleopods. Lateral spines of F′ have been cut to show their relative position. The scale is given by one millimeter slash marks. The cincinnuli, *cin,* are approximately 50 μ in length.

represented by a small flap or lobe on the distomedial edge of the endopodites. The lobe is free of cincinnuli (Fig. B). The appendices masculinae of the second pleopods (Fig. B′) are now almost half the length of the appendices internae.

The four apical spines that are typical of all appendices masculinae of larger forms are now present; however, they are much smaller (200 microns) than the spines of larger forms.

Stage 3. This stage is intermediate between 2 and 4 and perhaps may be skipped entirely in the developmental process since only a relatively small number is seen during the year. Individuals measuring 2.0 to 2.2 cm in May and June appear to be in Stage 3. The copulatory organs of the first pleopods are similar to those of Stage 2, however the appendices masculinae of the second pleopods are more than half the langth of the appendices internae. There is also a row of short spines that proceed obliquely down the ventral surface of the ramus, in addition to the four distal spines.

Stage 4. This stage represents individuals that range in size from 2.5 to 2.9 cm from November to April; and from 2.3 to 2.8 cm from May to September. This stage is typical of the summer non-reproductive males. The copulatory organs of the first pleopods now have a small number of cincinnuli at the apical end (Fig. D). The appendices masculinae are almost as large as the appendices internae (Fig. D′). In addition to the four apical spines which are now 500–600 microns in length, there is a row of six spines that run obliquely down the medial edge of the ramus. One or two spines may also be present on the ventral surface.

Stage 5. During the months of May through August this stage is found in the individuals measuring 2.9 through 3.5 cm that are producing sperm and functioning as males. For the most part those in this size range remain at this stage until late February and April when they attain Stage 6. Most of these individuals will function as males the following summer. Some, however, transform into females and into Stage 8 during March and April. The copulatory organs of the first pleopods are well developed and each has the appearance of a thumb-like projection from the disto-medial surface of the endopodite. There are many cincinnuli present at the apical end. The appendices masculinae are as large as or slightly larger than the appendices internae. There are from fifteen to eighteen broad spines present at the apical end and along the ventral surface. The largest of these spines has a length of from 700 to 800 microns. During January, the appendices masculinae of individuals of Stage 5 have shorter spines, measuring 300–400 microns. Probably a molt has occurred during November or December.

Stage 6. This stage represents the summer males measuring 3.5 to 4.2 cm that are functioning as males for the third summer. This stage first appears during February and March in the 3.1 through 3.8 cm size class males. The peak of development is reached from August to November. By January it appears that all of these individuals have molted and reached Stage 8. The copulatory organs of the first pleopods (Fig. F) are broad and triangular and each contains many cincinnuli along the medial margin. The appendix masculina (Fig. F′ of the second pleopods is at least 1½ times as long as the appendix interna and contains 18 to 23 spines at the apex and along the ventral surface.

Stage 7. After undergoing molts during the months of December and January, the individuals measuring 3.4 to 4.3 cm are in the process of transforming from males into females. The copulatory organs of the first pleopods are now beginning to regress (Fig. G). The apex is free of setae and the organ itself is once more stalked and contains less cincinnuli than the previous stage. The medial end of the endopodite is free of large setae, only very small stub-like setae remain. The appendix masculina (Fig. G′) contains very reduced spines and its length is nearly equal to the appendix interna.

Stages 8 and 9. These two stages are actually variations of the same stage. The copulatory organs are typical of those individuals above 3.5 cm carapace length that have transformed into females. This stage first appears after the May or spring molt. The animals are now fully transformed individuals. The copulatory organ of the first pleopod (Fig. H) has atrophied to a widened stump on the inner surface of the endopodite. The distal end of the endopodite has become pointed and displays small setae along its margin. The small spines that were present in the male along the inner margin have disappeared. The appendix masculinae also has begun to atrophy. It may be present as a ramus that is almost the length of the appendix interna or it may be reduced to a small papilla which displays one or two small plumost setae (Fig. H′). In *Pandalus platyceros* this rudiment of the appendix masculina may be present in the ovigerous female. Although the appendix interna does not increase in length in the female stage, it becomes more robust, increasing in width by almost one millimeter. The cincinnuli of the appendix interna of the female do not disappear; they, in fact, appear to increase in number. In addition to these changes, the pleura of the abdominal segments lengthen at this stage to cover the pleopods and eventually the attached eggs.

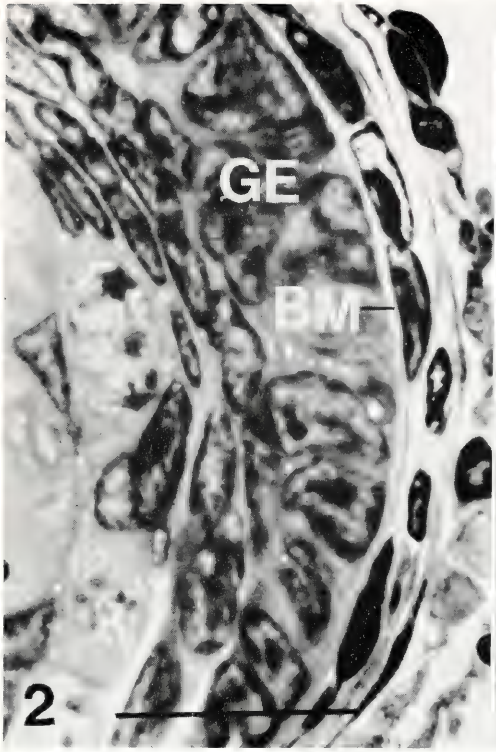
The gross morphology of the gonad and accessory ducts

The gonad of *Pandalus platyceros* in the dorsal thoracic hemocoel consists of a pair of tube-like arms that are joined by a transverse bridge of tissue one-third the distance from its anterior end. In addition, the posterior ends of the arms are connected to each other by sheaths of squamous cells that encapsulate each arm of the gonad. Three-fifths from the anterior or distal end, along the lateral surfaces, a pair of sperm ducts take their origin. In immature males the ducts are relatively uncoiled and each terminates at the male gonopore at the base of the coxa of the eighth thoracic appendage. In mature males, the sperm ducts can be further differentiated into three regions. Proximal to the gonad is the tightly coiled efferent tubule which comprises approximately 25% of the total length. Next there is a muscle-sheathed sperm duct proper which gradually increases in diameter as it approaches the distal end, the highly muscular ejaculatory bulb.

During the male stages, the ovotestis has a central medulla of ovarian tissue and a cortex of testicular tissue. Only during the female stages does the gonad become a true ovary lacking cortical testicular tissue.

A pair of thin-walled oviducts originate about half way from the anterior end of the gonad, just anterior to the origins of the sperm ducts. Each proceeds ventrally from the origin to insert on the gonopore which is located on the base of the coxa of the sixth thoracic appendage. The oviducts are present during the male stage although they are inconspicuous and difficult to distinguish from blood vessels.

The ovotestis extends anteriorly to the first or second thoracic segment near the dorsal projection of the cardiac stomach and posteriorly beneath the pericardial



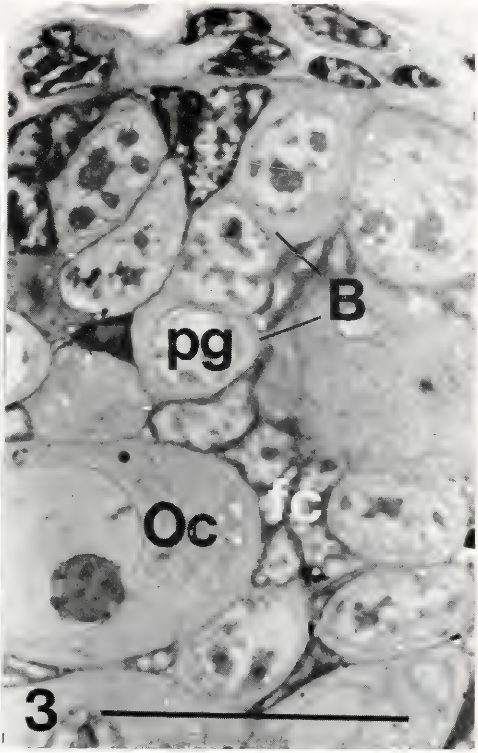


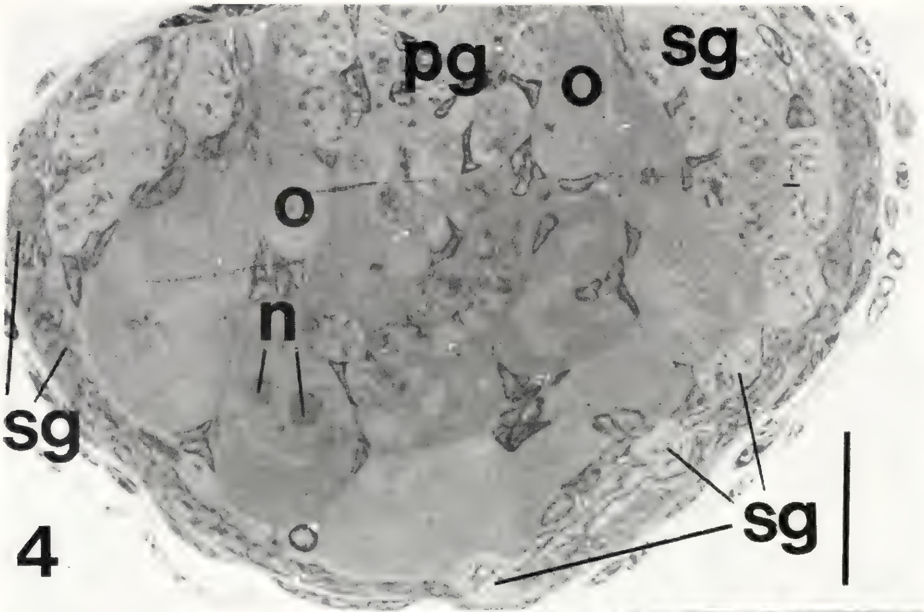
FIGURE 2. Transverse section (2 μ) through the gonad of a 2.3 cm CL male (October) showing gonodal epithelium (GE) and its basement membrane (BM). The slash mark represents 20 microns; glutaraldehyde-osmium fixation, Richardson's stain.

FIGURE 3. Transverse section (1 μ) through the ovarian germinal ridge of a 2.4 cm CL male (December). glutaraldehyde-osmium fixation, Richardson's stain. The slash mark represents 30 microns; B, intercellular bridges, fc, follicle cells, Oc, primary oocyte, pg, primary oogonial cells.

septum to the eight thoracic segment. A large hepatopancreas supports the gonad ventrally. During the latter stages of vitellogenesis, the ovary nearly fills the dorsal thoracic hemocoel, depressing the hepatopancreas and extending anteriorly into the subrostral hemocoel and posteriorly into the first abdominal segment.

The dorsal and lateral surfaces of the anterior half of the male stage gonad contains many red pigment cells or erythrophores in the capsular sheath. Whereas the posterior dorsal surface that lies beneath the pericardium usually remains unpigmented. During the female stage, in addition to a large number of erythrophores, there are numerous white pigment cells or guanophores in the sheath imparting a milky pink color to the gonad. Although the cells of the male stage gonad generally remain translucent and colorless, the olive green color of the eggs can be seen through the pigment cell layer of the ripe ovary.

Both ovotestis and ovary are anchored to the posterior lateral walls of the thoracic pleura by a pair of mesenteric sheaths that underlie the pericardium and overlie the posterior surface of the hepatopancreas. The anterior end of the gonad



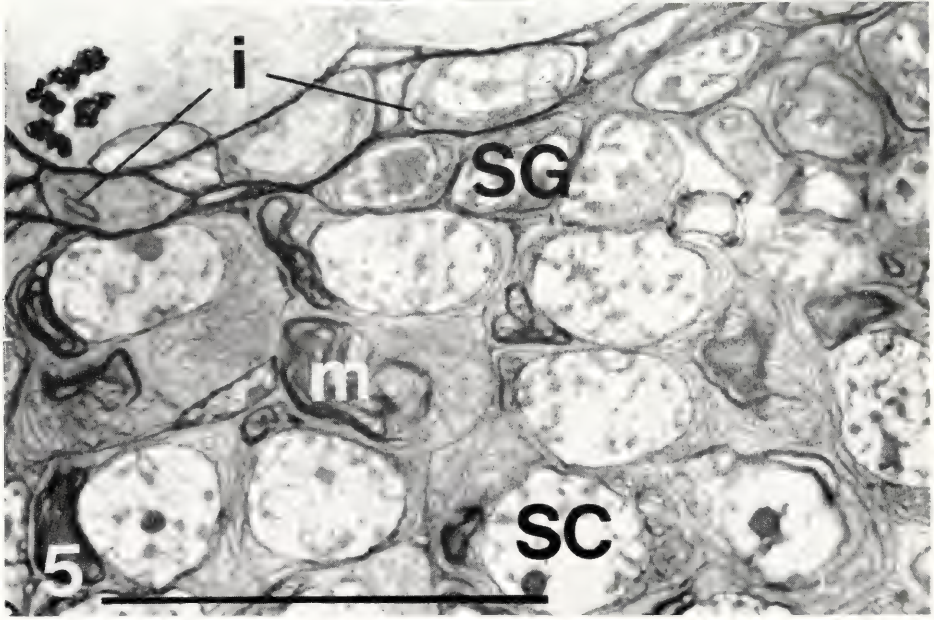


FIGURE 4. Transverse section (1 μ) through the ovotestis of a 2.4 cm CL male (March). Note that the primary oogonial cells (pg) are separated from the spermatogonial cells (sg) by the oocytes (o) that remain attached to the basement membrane of the gonodal epithelium by stalks; glutaraldehyde-osmium fixation, Richardson's stain. The slash mark represents 50 microns, n, nucleoli of oocytes.

FIGURE 5. Transverse section (1 μ) through a region of the cortex of the ovotestis of *Pandalus platyceros.* Note the well developed cytoplasmic inclusions (m) in the young

is attached to the anterior rostral region of the cephalon by strands of connective tissue that underlie masses of lymphogenous tissue. In addition, the saddle shape heart adds further support by sending a number of blood vessels over and into the gonad.

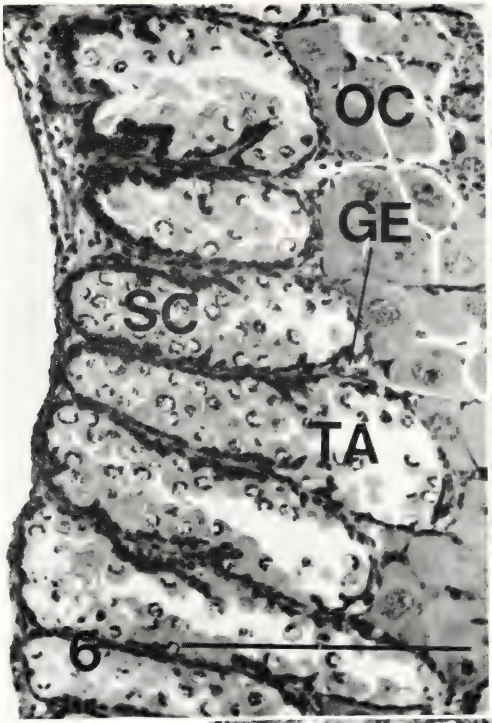
Development of the ovotestis and formation of the germinal ridge

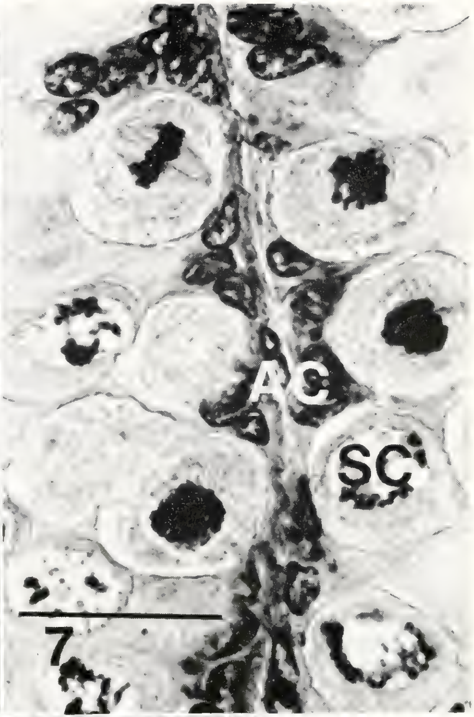
Each gonadal arm has a cortical layer of epithelial cells from which all other gonadal cells originate. In five to eight month old individuals this layer may be several cell layers thick and containing large, irregularly shaped and densely staining nuclei (Fig. 2). Because of the paucity of cytoplasm in this layer and the apparent absence of cell boundaries, the nuclei of this germinal epithelial layer appear to reside in a syncytium.

Peripheral to the germinal epithelial layer, there may be several layers of highly squamous cells. These cells appear to be an epithelium which also contains the numerous pigment cells. They are separated from the gonad proper by the basement layer of the germinal epithelium (Fig. 2). It appears that this cellular layer may be formed extrinsic to the gonad, at least in the initial stages of male gonad development.

By the time a shrimp reaches a carapace length of 1.7 cm each arm of the gonad is composed of a cortex of cells that is derived entirely from the gonadal epithelium and a medula of primary gonial cells. These primary gonial cells differentiate into the follicle cells and oocytes to form the ovarian portion of the gonad. The medial gonadal epithelium or the germarium proper gives rise to these medullary cells. The medulla is characterized by at least two cell types. The first cell type is similar in morphology to the cells of the gonadal epithelium. Cells of this type are the ovarian follicle cells. They are generally irregular in outline and consist mainly of nucleus with dense chromatin granules (Fig. 3). These cells appear to originate both from the germarium proper and also from the regions of the gonadal epithelium that surround the medulla. They do not appear to undergo any mitotic activity but simply move off the basement membrane and into the ovarian medulla where they become associated with the oocytes. The second morphological cell type is the primary gonial cell which also appears to be formed from the dense nuclear cells of the germinal epithelium (Fig. 3). Mitotic figures are rare in these cells. Increases in both nuclear and cytoplasmic volume occur after these cells move off the basement membrane of the epithelium. At this stage the cells have an average diameter of 15 microns. Many of these cells appear to have intercellular bridges. Their nuclei have a tendency to become highly lobated with large numbers of nucleoli and densely clumped chromatin. A few of the primary gonial cells do not lose contact with the basement membrane and become flask-shaped. These cells move laterally as more gonial cells are proliferated from the germinal ridge or germarium. However they still retain their connection with the basement membrane by a slender neck of cytoplasm (Fig. 4). With the further increase in cytoplasmic volume the nuclei have many large nucleoli. At this stage of

spermatocytes (SC). Also note the early development of these inclusions (i) in the spermatogonial cells (SG); glutaraldehyde-osmium fixation, Richardson's stain. The slash mark represents 50 microns.





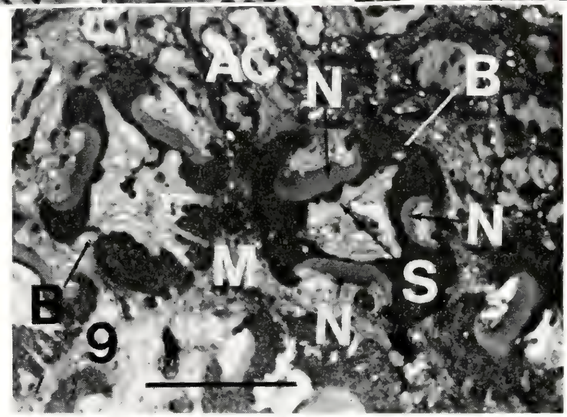


FIGURE 6. Frontal section (6 μ) through the ovotestis of a 3.6 cm CL male (June). Note the subdivision of the testicular cortex into a number of acini (TA) by the evagination of the gonodal epithelium (GE). The inner portion of this epithelium forms the lateral germinal ridge which gives rise to the spermatogonia; Hematoxylin–Halmi stain. The slash mark represents 500 microns, OC, oocytes, SC, spermatocytes.

FIGURE 7. Frontal section (6 μ) of a region of the testicular cortex of the gonad of a 3.1 cm CL male (June). Note the acinar cells (AC) projecting into the acinar lumina which are filled with spermatocytes (SC) undergoing synchronous maturation activity. Azan stain. The slash mark represents 50 microns.

FIGURE 9. Frontal section (1 μ) through two tetrads of spermatids in a testicular acinus of *Pandalus platyceros*. Note the three spermatids joined together by intercellular bridges (B).

development, the cells become primary oocytes and may measure 30–40 microns in diameter. As the cytoplasmic volume of the oocytes increases, the cell moves deeper into the ovarian medulla although still attached by the stalk to the basement membrane (Fig. 4). Finally, the stalk appears to break and the oocyte takes up a position in the peripheral region of the medulla. The primary gonial cells that appear to dissociate from the germinal ridge comprise the central region of the ovarian medulla. These cells possess nuclei in various stages of mitotic prophase and thus have yet to differentiate into primary oocytes. In addition, there are numerous cells with lobate nuclei which appear to be in association by intercellular cytoplasmic bridges with each other and with primary oocytes (Fig. 3). These cells may be nutrient or nurse cells. Whether or not these cells differentiate into oocytes could not be determined through histological sections.

In those individuals that attain a carapace length greater than 2.2 cm by November of their first year, there is cellular differentiation in the gonadal epithelium lateral to the ovarian germinal ridge. As in the case of the oogonial cells, the cytoplasmic volume of these lateral cells begins to increase and the nuclei take on a lobate shape. These cells do not remain attached to the basement membrane but move into the tube or arm of the gonad lateral to the ovarian medulla. The cytoplasm of these cells contains dense staining inclusions that have the appearance of microtubules in glutaraldehyde-osmium fixed tissue (Fig. 5). These cells appear to be the spermatogonia. Once they have taken their position within the cortical region of the gonad, most of these cells undergo asynchronous mitotic division into secondary spermatogonial cells. By this stage the nuclei of the cells are in various stages of mitotic activity. Many of these cells will divide again, and with each division will move more laterally.

After the mitotic divisions are completed, the cells have increased in size to 40 microns. The swirls of microtubules have increased to fill the cytoplasm (Fig. 5). These cells have now become primary spermatocytes since no mitotic activity is evident. Many of these primary spermatocytes appear to be connected to each other, perhaps by remnants of spindle fibers. The chromosomes appear to be in a pre-leptotent stage. No nucleoli are evident. The nucleus reaches a maximum diameter of 40 microns; the cell itself reaches a diameter of 50 to 60 microns. When primary spermatocytes are formed by first year individuals during the winter and early spring, these germ cells degenerate and a new crop of primary spermatocytes take their place. Individuals of less than 2.2 cm carapace length in November appear not to produce primary spermatocytes until late summer (August–September).

With the formation of spermatogonia from the lateral germinal ridges, there is a lifting or elevation of the lateral edges of the gonodal tube. The dorso-medial surface that comprises the ovarian germinal ridge appears to be held down by the flasked shaped oocytes that have the major portion of their cell masses anchored within the medulla of the gonad. The ovarian germinal ridge also begins to protrude outward due to the mechanical pressure of the uplifting of the lateral edges,

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The fourth spermatid of each tetrad is out of the plane of section. Note also that the spermatid tetrads are surrounded by mucous (M) secreted by the acinar cells (AC); glutaraldehyde-osmium fixation, Richardson's stain. The slash mark represents 25 microns, N, nucleus of spermatid, S, developing spine of spermatozoon.

TABLE I

The relationship between age and carapace length to state of gonadal development in Pandalus platyceros *Brandt*

Age in months	Sex	Carapace length (cm)	State of gonadal development
0– 5 (Apr.–Aug.)	—	Planktonic larvae	----------------------------------
5– 7 (Sep.–Nov.)	Imm.	1.2–2.2	Oogonia and oocytes; no spermatogonia
7–14 (Nov.–Jun.)	♂	1.7–2.2	Oogonia and oocytes; few spermatogonia after Jan.
7–16 (Nov.–Aug.)	♂	2.2–2.8	Spermatogonia and spermatocytes present; average oocyte 60 μ
13–16 (May–Aug.)	♂	2.9–3.5	Spermatids present; spermiogenesis; average oocyte 100 μ
16–19 (Aug.–Nov.)	♂	2.7–3.3	Spermatogonia and spermatocytes; no spermatids; average oocyte 120 μ
16–19 (Aug.–Nov.)	♂	3.3–3.7	Testicular tissue degenerates; increase in oogonia and oocytes, average oocyte 250 μ
19–24 (Nov.–Apr.)	♂	3.0–3.8	Increase in proliferation of spermatocytes; average oocyte 130 μ
19–24 (Nov.–Apr.)	Trans.	3.5–4.0	Increase in proliferation of oocytes and primary oocyte growth; average oocyte 370 μ
24–28 (Apr.–Aug.)	♂	3.5–4.0	Spermatids present; spermiogenesis; average oocyte 180 μ
24–28 (Apr.–Aug.)	♀	4.2–4.5	Increase in oocyte growth with deposition of proteinaceous and lipid yolk platelets; average oocyte 900 μ
28–31 (Aug.–Nov.)	♀	3.7–4.2	Testicular tissue degenerates; increase in oogonia and oocytes; average oocyte 250 μ
28–31 (Aug.–Nov.)	♀	4.5–4.9	Spent ovary; ovigerous females

now called lateral wings of the gonad. The lateral wings are formed because of the increased production of spermatogonia and spermatocytes which move out laterally as more spermatogonia are produced from the lateral germinal ridges. In addition, there are formed a series of lateral evaginations of the dorso-lateral gonadal epithelium which compartmentalizes the gonodal tube into a series of cortical testicular acini (Fig. 6). These evaginations greatly increase the surface area of the lateral germinal ridges which result in an increased production of spermatogonia. In addition, the infoldings and evaginations of the lateral germinal epithelium causes the ovarian and testicular germinal ridges to take up a central position within the gonadal tube, completely surrounded by the testicular acini. Although the lateral germinal ridges of the gonadal epithelium form most of the spermatogonial cells, some spermatogonial cells may be found along the basal part of the gonadal epithelium.

There are two age classes of males present during the winter and spring months (Table I). The first age class that hatched the previous year during April and May consists entirely of males. Most of these shrimp will function as males during the coming summer, only those below 2.2 cm in carapace length in January that have not yet produced any spermatogonia will not produce spermatozoa during their second summer. Their first summer is spent in the plankton as pelagic zoea larvae. The second age class is composed of shrimp that hatched during the spring two years previously. Most of the shrimp below 3.5 cm in January will function as males during the coming summer. Those above 3.5 cm and a very few between 3.2 and 3.5 cm are in a transitional stage and will function as females in the coming summer.

During the winter months, growth nearly is arrested in both age classes of *P. platyceros*. Similar conditions have been reported for *Pandalus kessleri* (Aoto, 1952) and *Leander serratus* (Forster, 1951). The gonads of specimens of *P. platyceros* below 2.2 cm carapace length show little indication of spermatogonia formation. However, by late March or early April, when growth is once more underway, spermatogenesis is initiated. In these same animals oogenesis began in early autumn and is still progressing since the primary oocytes have grown to an average diameter of 50 microns. Usually these oocytes degenerate in these immature forms. In those individuals above 2.2 cm of the first age group, spermatogonia are evident in the cortex of the gonad as early as November of the previous year. Thus, these individuals are about seven months old when spermatogonia formation is initiated. By January, a 2.4 cm individual, the cortical region of the gonad is filled with spermatogonia and spermatocytes and the medullar oocytes have attained an average diameter of 60 microns. By the end of June, a 2.9 cm shrimp has large oocytes with an average diameter of 100 microns.

Although primary spermatocytes are abundant in the testicular acini of first year age group during the winter and spring months, the maturation divisions have yet to occur. The nuclei of the spermatocytes are arrested at the leptotene and zygotene stages of prophase. As new populations of spermatogonia and spermatocytes are produced, the oldest spermatocytes undergo degeneration and younger spermatocytes take their place in the acini. At this time the gonodal epithelium that surrounds the testicular acini, the acinar cells of the testis, is composed of highly squamous cells with flattened nuclei. These squamous cells are characteristic of the gonodal epithelium of the testis during the nonreproductive seasons.

Almost all individuals that have a carapace length less than 2.5 cm in June do not produce sperm during the summer months; however, they will become reproductive during the following summer. By September, these same individuals reach a maximum carapace length of 3.1 cm and their sperm ducts contain no spermatozoa.

Maturation of the spermatocytes and spermiogenesis

The actual time of sperm formation is directly related to the size of the shrimp. The larger the male the earlier sperm formation begins. Males entering their third spring initiate the maturation processes as early as April. Those second year males that produce spermatocytes the previous autumn and winter begin to produce secondary spermatocytes as early as May. Also, any second year males that have not produced spermatocytes until early spring generally do not demonstrate meiotic spermatocytes until late July or early August. By September the males with a carapace of 2.7 cm or less, do not produce sperm until their third summer when their carapace length ranges from 3.5 to 4.0 cm (Table I).

The first indication of testicular meiotic activity is the proliferation and hypertrophy of the acinar cells of the testis. The cortex of the testis becomes greatly distended with spermatocytes which in turn appear to form an association with the acinar cells which project into the acini (Fig. 7). Wave after wave of spermatogonia are produced. Finally, only primary spermatocytes fill the acini. No longer

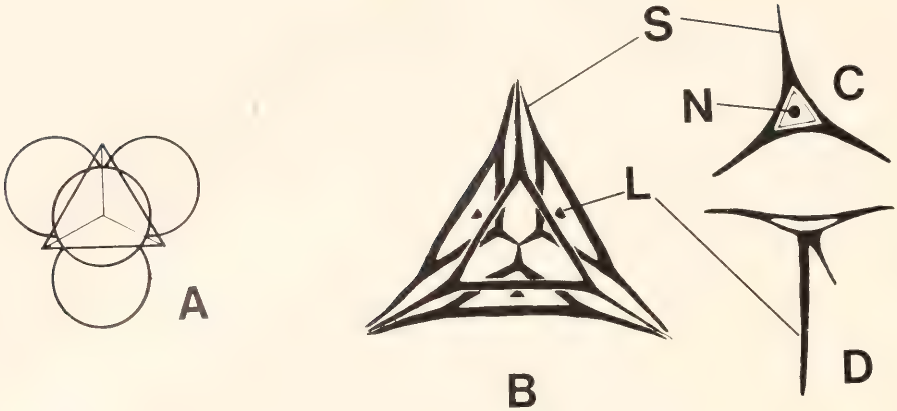

FIGURE 8. Spermiogenesis in *Pandalus platyceros* Brandt; (A.) the tetrahedral configuration of daughter spermatids from the same mother spermatocyte; (B.) diagrammatic representation of the origin of the triangular shaped spermatozoon based on the tetrahedral arrangement of daughter spermatids. The developing large spine, L, projects inward; s, short lateral spines; (C.) frontal view of a spermatozoon with the large spine projecting from the page; n, nucleus; (D.) side view of the spermatozoon. The scale is given by the large spine which is 40 microns in length.

are any spermatogonia produced by the germinal ridges. This cessation in the formation of spermatogonia coincides with the change in morphology of the acinar cells. The first meiotic division approximately is synchronous. The second meiotic division closely follows the first; in fact, actively dividing primary and secondary spermatocytes are evident in the same acinus. The spermatids that are derived from the same mother spermatocyte will remain in association with each other. During the latter stages of meiosis, nuclear condensation occurs with the extrusion of nucleoplasm into the surrounding medium. The four spermatids joined by intercellular bridges form a tetrahedron with each of the flattened nuclei forming the sides of the tetrahedron (Fig. 8). Fawcett, Ito and Slautterback (1959) demonstrated that cells such as spermatocytes that undergo synchronous division are connected to each other by such intercellular bridges. At this stage the spermatid tetrads are deeply surrounded by the acinar cell mucous (Fig. 9). The spermatid begins to flatten out, each forming a triangular side of the tetrahedron. The cytoplasm secretes a chitinous sheath surrounding the highly flattened nucleus. The tips of each triangular shaped cell project outward to form small spines. A large chitinous spine ultimately measuring 40–50 microns develops from the inner side of the spermatid. As each of the large spines of the four maturing spermatozoa grow inward they probably disrupt the tetrahedral arrangement. At this stage the acinar cells begin to regress to form a cuboidal epithelium lining each acinus. The lumen of each acinus is then filled with spermatozoa and mucous which is highly PAS positive and also paraldehydefuchsin positive. Finally, the acini evacuate their contents into the sperm ducts which are continguous with the testicular acini. It appears that the acinar cells are similar in function to the mammalian prostatic cells in that their product functions in the evacuation and perhaps maintenance of the immotile spermatozoa from the testis into the sperm ducts.

The transformation of the male gonad

Once spermiogenesis is completed and the sperm ducts are filled with spermatozoa, there is a tendency for the males to initiate the transformation into the female phase. The testicular gonadal epithelium (acinar cells) has lost its ability to form more spermatogonia since it has differentiated into mucous producing cells during spermatogenesis. Post-spermatic males may initiate gonadal transformation either when they are 16 to 19 months old, or, if they did not produce spermatozoa during their second summer, when they are 28 to 31 months old (Table I). As early as late July the testicular epithelial cells begin to degenerate and form scar-like connective tissue. The lumina of the acini are obliterated by newly formed and growing primary oocytes which push the degenerating testicular tissue against the squamous tissue sheath of the gonad. This atrophying testicular tissue forms the thick outer sheath of the female gonad. At the same time, many white chromatophores make their appearance in this outer sheath along with the numerous red chromatophores that were present during the male stage. Most of the large oocytes (100–200 microns) that were formed during the male stage are also in a state of degeneration and appear to be phagocytized by their surrounding follicle cells. Baffoni (1960) reported that the follicle cells of female decapod gonads do phagocytize oocytes following ovulation. The areas left by the degenerating oocytes are soon filled by new primary oocytes proliferating from the median germinal ridge.

After the sperm ducts have filled with spermatozoa and mucous, the epithelial layer of the ducts become tall and columnar. Paraldehydefuchsin granules appear in the cytoplasm and ultimately the cells secrete a cuticular sheath around the sperm mass and then regress to form a highly squamous epithelium. During copulation, the outer circular muscle of the ducts contract forcing a cuticular enveloped strand of sperm through the gonopores.

By November, all of the transforming shrimp have the external sex characteristics of functional males (stages 5 and 6). These male characteristics are maintained during the winter months relatively unchanged, but the gonads during this time are totally ovarian. Thus the shrimp have the primary sex organs of true females but the external secondary sex traits of males.

Discussion

The gonads of the higher crustaceans are similar to those of mollusks in that both are covered by a germinal epithelium (Raven, 1961). There is some confusion in the early literature as to the role of this epithelium in the production of gonial cells. Grobben (1878) noted an epithelial coat on the gonads of all the male decapods that he studied. He believed that certain regions of this epithelium gave rise to the male germ cells. The remainder of the epithelium has the function of secreting the spermatic plasma that envelops the mature sperm. Because the germinal region which he called the "Ersatzkeim" contained so many densely packed nuclei of irregular outline and form he described it as a syncytium. He even believed that many of these nuclei were undergoing amitoses. This statement was based on nuclei which were dumbbell shaped and thus appeared to be splitting in two. However, since the nucleus itself is not a rigid structure but rather a simple structure surrounded by a double membrane, the tight packing of these nuclei into a tubular structure would cause them to become highly distorted. Such distorted nuclei are present in the gonadal epithelium of *P. platyceros* especially during the juvenile male stages when the nuclei are stratified.

Gilson (1884) also stated that the "metrocytes" or sperm mother cells appear to be formed from the "Ersatzkeim" or replacement blastema. He also described this region as a synctium, however, he used the term plasmodium which is really not applicable to animal tissue. From both wax and thick epon sections it is difficult to disprove that the replacement blastema is a synctium. But this layer does possess a basement membrane which is typical of an epithelium. Because of the paucity of cytoplasm surrounding the nuclei, the gonadal epithelium and replacement blastema may appear to be a syncytium.

Another observation of both Grobben (1878) and Gilson (1884) was the lack of mitotic figures in the gonadal epithelium and especially in the region giving rise to the spermatogonia. I also have not found a preponderance of mitotic figures present in the epithelial layer and especially the germinal ridge (replacement blastema). However, mitotic figures are infrequently found in the germinal epithelium of *P. platyceros*. The paucity of such figures may indicate a rapid rate in the mitotic cycle. This epithelial layer is reminiscent of the androgenic gland tissue which also has a proclivity toward unseen mitotic activity. Sabatier (1885) reported that the spermatocytes which he called protospermatoblasts are derived from the connective tissue layer that forms the skeleton of the tubule in decapods. The epithelial layer of the gonad of *P. platyceros* is separated from the squamous tissue sheath of the gonad by a basement membrane. It, therefore, is improbable that such a layer of cells could produce spermatogonia into the lumen of the gonad. Initially, the squamous tissue sheath of the gonad appears to be formed extrinsic to the gonad perhaps by wandering scleroblasts.

The gonads of natantian decapods resemble those of amphipods more than other decapods. Meusy (1963) also noted in *Orchestia gammarella* that the zone of proliferation of spermatogonia is limited to a certain region of the gonadal epithelium. This germinal ridge appears to be a syncytium according to Meusy.

In protandric mollusks such as *Crepidula*, the entire germinal epithelium alternatively first gives rise to male and then female sex cells (Coe, 1936). But in a protandric hermaphrodite, such as *Pandalus* in which both male and female gametes can be produced simultaneously, there are questions remaining on the mechanism of the production of these two cell types. Aoto (1952) working on *P. kessleri* stated that the germinal ridge that runs down the medio-lateral surface gives rise to oogonia whereas the spermatogonia have a different origin within the interstitial cell masses that encapsulate the central ovarian mass. These interstitial cell masses are similar to the lateral germinal ridges of *P. platyceros* following the uplifting of the testicular acini. Berreur-Bonnenfant and Charniaux-Cotton (1965) observed in *P. borealis* that there is one germinal ridge per gonadal tube which alternatively gives rise to spermatogonia and oogonia. They explain this alternation in germ cell production as being due to a difference in androgenic hormone titer. When the titer is high, spermatogenesis occurs; when it is low, oogenesis. The observations on *P. platyceros* do not corroborate these observations. There are two distinct germinal areas that run down the gonadal tube. Each ridge is separated at all times from the other by large primary oocytes that remain attached to the basement membrane until others generally take their place. Although such stalked oocytes are evident in the drawings of Berreur-Bonnenfant and Charniaux-Cotton (1965), they do not attach any significance to them. Such histological data present in their paper is used to define a physiological manifestation; i.e., change in hormone titer which this author feels is presently undefinable due to lack of a reliable bioassay (Hoffman, 1969). In addition, no data is given as to the age or season of the year of the shrimp used in their analyses. Alternation in oogenesis and spermatogenesis from the medial germinal ridge has never been observed in male stage of *P. platyceros* under natural conditions. Both phenomena appear to occur simultaneously. During the adult male stages there is an increase in the number of spermatogonia that are proliferating from the lateral germinal ridges, but oogenesis is still occurring, albeit not at the same rate as spermatogenesis. Oogenesis is a continuous process in *P. platyceros* since young oogonia are always present in the ovarian medulla of male individuals. This observation is similar to the observations made on the ovotestes of hermaphroditic mollusks such as *Helix* (Ancel, 1903; Gatenby, 1917).

Of important note is the large size of the primary spermatocytes of *P. platyceros*. It is relatively rare that animal spermatocytes ever attain a diameter of 40 to 60 microns. I believe that these cells actually may be oocytes modified through the action of a male hormone from the androgenic glands. If we assume that protandry in pandalids arose from gonochoristic stock, natural selection would tend to favor the female stages as the originator of the protandric evolutionary line. The male stage could be considered as the "juvenile" stage when little energy would be necessary for the formation of sperm. In addition, male stages could be smaller than females since they require little volume to produce and store sperm. The female then would be the "adult" stage. A large organism is required to produce a large number of large yolky eggs. Also, a large animal has a selective advantage especially when it must carry its fertilized eggs on its abdominal appendages over a period of several months. Berreur-Bonnenfant (1963) demonstrated that testicular cells of certain Malacostraca when cultured *in vitro* in the absence of androgenic glands will autodifferentiate into oocytes. Also, Charniaux-Cotton (1957) observed the anlage of androgenic glands in female talitrid amphipods. However, these anlage never become functional in the female. Therefore, female malacostracans do have the potential to differentiate into males. Through a series of exciting experiments Charniaux-Cotton (1959) has been able to reverse the sex of female talitrid amphipods by the implantation of androgenic glands. These experimentally reversed females not only produce viable sperm but will even copulate with other females. It could be argued that protandry arose from the male line instead of the female, but then such males would have had to develop a very complex series of mechanisms for the development and maturation of oocytes. This is something that is already innate in the female when the ability of some malacostracan oocytes to autodifferentiate is taken into account (Berreur-Bonnenfant, 1963).

Legrand (1964) has proposed the concept of gonad territory to explain how a single gonad might give rise to both male and female sex cells. In oniscoid isopods, Legrand observed that the axial part of the gonad gives rise to oogonia, while the utricular part forms the spermatogonia. The androgenic hormone only then would have the function of initiating the development of the testicular utricles into which the germ cells migrate after they are first formed in the axial tube. A territoriality such as this may be set up in the germinal ridge of *Pandalus* subdividing it into a medial ovarian region and lateral testicular regions by the primary oocytes that have been formed in the early stages of gonad development before there had been any production of spermatogonia. Shrimp in their first autumn (five to eight months old) measuring 1.2 to 2.0 cm in carapace length have oogenesis well underway, although the oocytes only reach a maximum diameter of 40 microns. Spermatogenesis has yet to occur. These immature forms could be considered more female than male. At this same stage of development the androgenic glands are in their initial stages of development (Hoffman, 1969). Since the proliferation of oogonial cells in the gonad appears to precede androgenic gland development, the medial ovarian germinal ridge may not be susceptible to androgenic hormone when it becomes present. This territoriality could have been introduced when the shrimp are from five to eight months old. If the androgenic glands do not develop, the gonads could produce oocytes and this way result in the formation of primary females that are evident in certain pandalid species (Butler, 1964).

I wish to thank the director of the Friday Harbor Laboratories, Dr. Robert L. Fernald, for the generous facilities afforded me while this study was undertaken. Also, many sincere thanks to Dr. Paul L. Illg for his most generous advice and criticism over the years of this study.

Finally, to *Pandalus platyceros* for always being available in sufficient quantities for the duration of this study.

SUMMARY

1. The relationship between age and carapace length to the state of gonadal development in *Pandalus platyceros* Brandt is presented. The development of the ovotestis is shown to be a function of size (carapace length) and not age.

2. The copulatory appendages of the pleopods appear to pass through seven or eight stages from the immature form to the adult female stage.

3. The development of the ovotestis is described with special reference to the origin of gonial cells. It appears that oocytes and spermatocytes derived from two distinct germinal areas of the gonadal epithelium. The lateral germinal ridges give rise to spermatogonia and acinar cells; the medial germinal ridge, oogonia and follicle cells.

4. The flattened triangular spermatozoa are derived from the tetrahedrally arranged tetrads of spermatids.

5. Transformation to the female stage generally is initiated only after the males have produced spermatozoa. The lateral germinal ridges appear to have lost their ability to form spermatogonia since they have differentiated into prostatic-like mucous producing cells.

6. In the transforming stage, the degenerating cortical testicular tissue forms the thickened outer sheath of the ovary. Four months after spermatozoa forma-

tion, the sperm ducts are filled with sperm, the copulatory organs are still masculine; but the gonad lacks testicular tissue and becomes a true ovary.

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ON THE ENDOGENOUS CONTROL OF TIDE-ASSOCIATED DISPLACEMENTS OF PINK SHRIMP, *PENAEUS DUORARUM* BURKENROAD[1]

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Extensive movements are carried out by pink shrimp during the course of their life cycle. In the population studied here the larval and postlarval stages move, or are displaced, from their offshore spawning sites within the Gulf of Mexico to inshore nursery areas within the Florida Everglades. They remain within these inshore waters for several months, during which they undergo rapid growth, before returning again, as juveniles, to deeper offshore waters.

It is now well established (Tabb, Dubrow and Jones, 1962; Hughes, 1969c; Beardsley, 1970; Roessler and Rehrer, in press) that the inshore and offshore movements of the postlarvae and juveniles, respectively, are facilitated by the selective use of tidal currents. From the sampling of consecutive tides in a canal leading into the Everglades, the above mentioned workers found that postlarvae were taken predominantly in flood tide samples, whereas juveniles were taken exclusively during the ebbing tide.

An earlier study (Hughes, 1969a) indicated the probable mechanisms whereby postlarval and juvenile pink shrimp discriminate between and utilize the tides for their inshore and offshore displacements. It was shown that the shrimp were able to perceive and respond appropriately to very small changes of salinity, well within the range of salinity change normally occurring between tides in the vicinity of the everglades. Although these results suggest the probable mechanism whereby tide-associated movements take place in this species, they do not preclude the possibility that other aspects of water quality, which change with change in tide, could similarly elicit behavior changes, enabling the use of tides for displacements.

There is also evidence that the behavior responsible for these displacements is under endogenous control (Hughes, 1969b). Both the direction of swimming (with respect to the current) and the velocity of swimming of migrating juvenile pink shrimp within a current chamber, showed a phase relationship with the tide cycle in the area from which the shrimp had been collected the previous day. This relationship appeared to be adaptive in so far as downstream swimming was confined to the time of the ebb tide. (In nature, juvenile shrimp, migrating out of inshore waters, are found almost exclusively within the water column of ebbing tides.) It was concluded that some aspect of the tide cycle to which the shrimp were exposed prior to capture entrains a pattern of swimming which may persist for at least two days in a current, constant in terms of speed and "water quality."

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From these results the nature and extent of this control was not clear. This paper describes a subsequent study aimed at elucidating the role played by internal rhythms in the movements of both postlarval and juvenile pink shrimp.

APPARATUS AND METHODS

Two identical current chambers were used in all experiments. These have been described before (Hughes, 1969a) and are modifications of the chambers used by Creutzberg (1961) in his study of tide-associated displacements of elvers. These chambers were maintained in a light-tight room, in which illumination was constant throughout all experiments and was provided only by single 10 w red bulbs (General Electric 10s 14 Batch NR), placed directly over each chamber. All other factors of the animal's environment were maintained as constant as possible. The water within the current chambers was not changed during the experiments, but was aerated continuously by means of a single airstone suspended a few centimeters above the substrate. The temperature of the water within the chambers increased gradually and evenly throughout each experiment, due to the presence of the observers in the darkroom and the heat given off by the motor driving the paddle systems. The initial temperatures were in the range from 26–28° C but over the three day period rose in each case by 2 or 3° C.

The postlarvae were subjected to a constant current velocity of 5.5 cm/sec, while the current velocity in the case of the juveniles was 7.5 cm/sec.

The shrimp were collected on appropriate tides from Buttonwood Canal within the Everglades National Park, Florida, using "Discovery" type plankton nets suspended into the current from a bridge over the canal. They were transported immediately to the laboratory, the journey taking approximately 2 hours and placed within the current chambers. In the postlarval experiment approximately 200 individuals were placed in each chamber; 10–12 individuals were used in the juvenile experiments.

Records of the movement of the shrimp within the chambers were initiated at midday on the day following collection and were continued for three days. In the case of the juveniles collected from a very early morning ebb tide, the experiments were initiated at midday of the day of capture. In all experiments the movements of the shrimp within the current chambers were recorded at half-hour intervals. They were recorded in terms of the number of shrimp swimming downstream and the number swimming upstream during a two-minute period.

The animals were not fed for the duration of the experiments, as feeding rhythms (Hughes, 1968) could have interfered with the rhythms under investigation.

RESULTS

Postlarvae

The results (Fig. 1) show a pattern of up- and downstream swimming in the current chamber which is clearly in phase with the tide-cycle in nature. Upstream swimming in the chambers is greatest at times of flood tide in the area of capture, whereas downstream swimming predominates at times of ebb tide. The validity of these results is strongly supported by the marked similarity of the pattern obtained in both current chambers. It is further verified by results (not presented

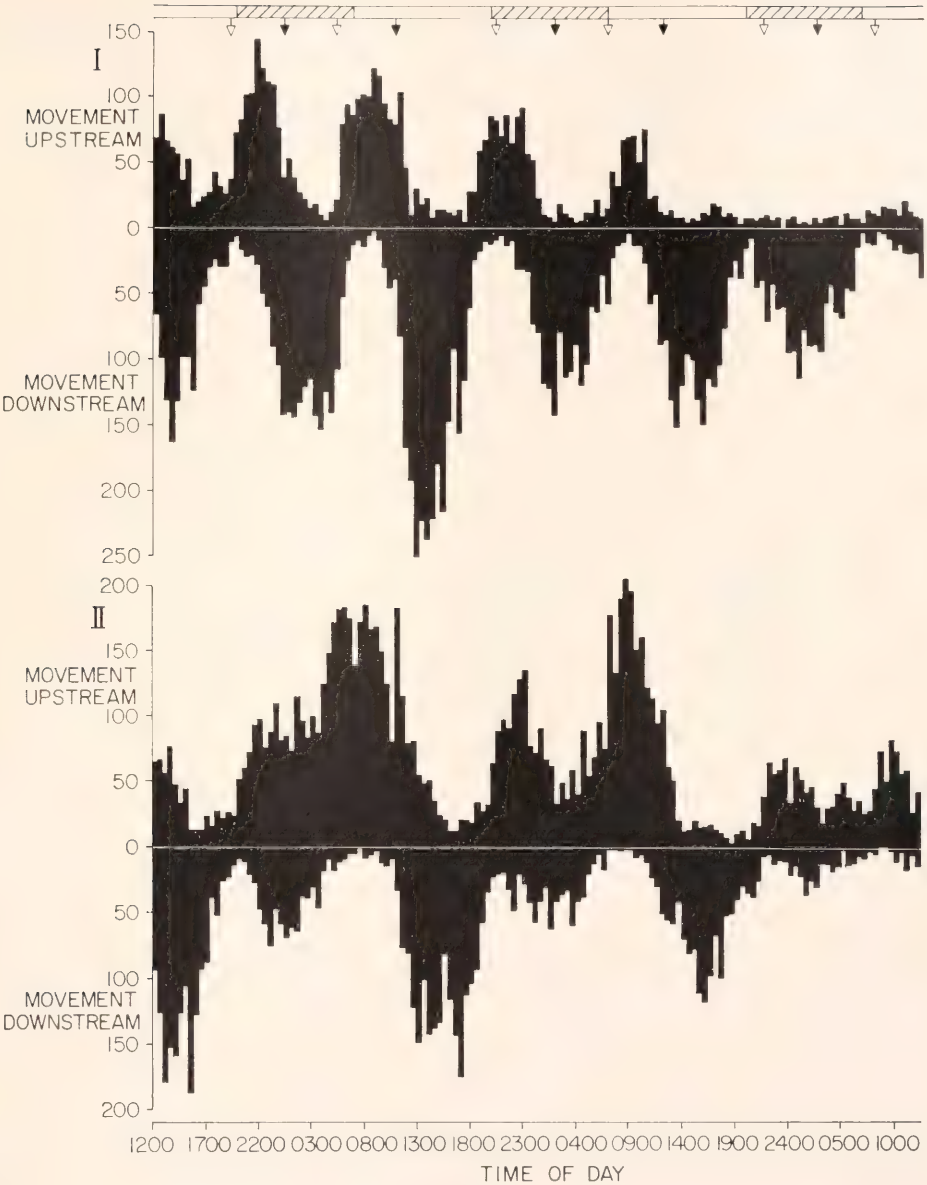

FIGURE 1. The pattern of swimming of postlarval shrimp maintained under constant conditions within two current chambers (I and II) for three days following their collection from nature. Swimming was recorded in terms of the numbers of individuals which moved in an up or downstream direction during two minute observation periods made at 30 minute intervals. The times of day and night in nature are represented by the open and transverse bars, respectively, at the top of the figure. Time of low and high tide in nature are indicated by the open and shaded arrows, respectively. (See text for description of constant conditions.)

here) of a preliminary experiment which had to be discontinued due to a malfunction within the current chamber system. Prior to discontinuation of that experiment, the animals showed peaks of up- and downstream swimming identical to those obtained in the experiments recorded here.

There is no indication from these results of circadian rhythmicity. The tendency to be active is apparently as great during the hours corresponding to "day" as during those corresponding to "night." The decrease in the level of activity which is evident by the third day is probably as much a function of starvation as a decline in the strength of the rhythm.

A high degree of endogenous control, in which the influence of both circadian and tidal rhythms may be discerned, is evident from these results (Figs 2 and 3). Endogenous control is evidenced by (i) the marked similarity of the direction and velocity of swimming of all individuals in the same tank (ii) the similarity of patterns of swimming obtained at all times in both tanks and (iii) the reproducibility of the pattern each day.

The nature of the relationship existing between the pattern of swimming and the tide regime from which the shrimp had been collected confirmed the results of previous experiments (Hughes, 1969b) in which it was found that shrimp, collected from an ebb tide occurring early in the evening (new and full moon), would, the following night in the current chamber, swim downstream for an interval which appeared to correspond to the duration of the ebb tide (Fig. 2). Thereafter they reversed direction and swam upstream for the remainder of the "night." The results of this most recent series of experiments show that this was not always the case but, by the time of the second and third "night," downstream swimming took place for a longer period and, sometimes extended even to the time of "dawn." Nevertheless, although it is not readily conveyed by the figures, it was usually apparent that the swimming behavior of the shrimp changed each night at about the time of the end of the ebb tide. Active downstream orientation diminished, often quite sharply, giving way to passive drifting and "indecisive" movements. Downstream displacement was scarcely diminished however, indicating that, although the juveniles actively orient in a downstream direction, like the postlarvae, they do not apparently swim very actively and their displacement downstream is largely a function of current velocity. Downstream swimming, which would be expected to occur during the subsequent early morning ebb tides, is absent, however, and only during the second "day" of Figure 2(II) is there the inactivity, characteristic of shrimp in nature (Fuss and Ogren, 1966), and which has been shown to occur in laboratory studies within stationary water under constant conditions (Hughes, 1968) and in current chambers when a day/night cycle is artificially imposed (Hughes 1969b).

The pattern of swimming of shrimp collected from an ebb tide occurring late in the evening (quarter moons) also confirmed earlier results (Hughes 1969b) insofar as, during the first night at least, the shrimp swam upstream throughout the night (Fig. 3). However, after the first night in the current chamber, these shrimp, when swimming, oriented almost exclusively downstream. The onset of the downstream swimming seemed to be determined by the time of the start of an ebb tide and was terminated the following day at apparently the time of dawn. Identical results (not presented here) were obtained when this experiment was repeated again with shrimp collected from a late evening ebb tide.

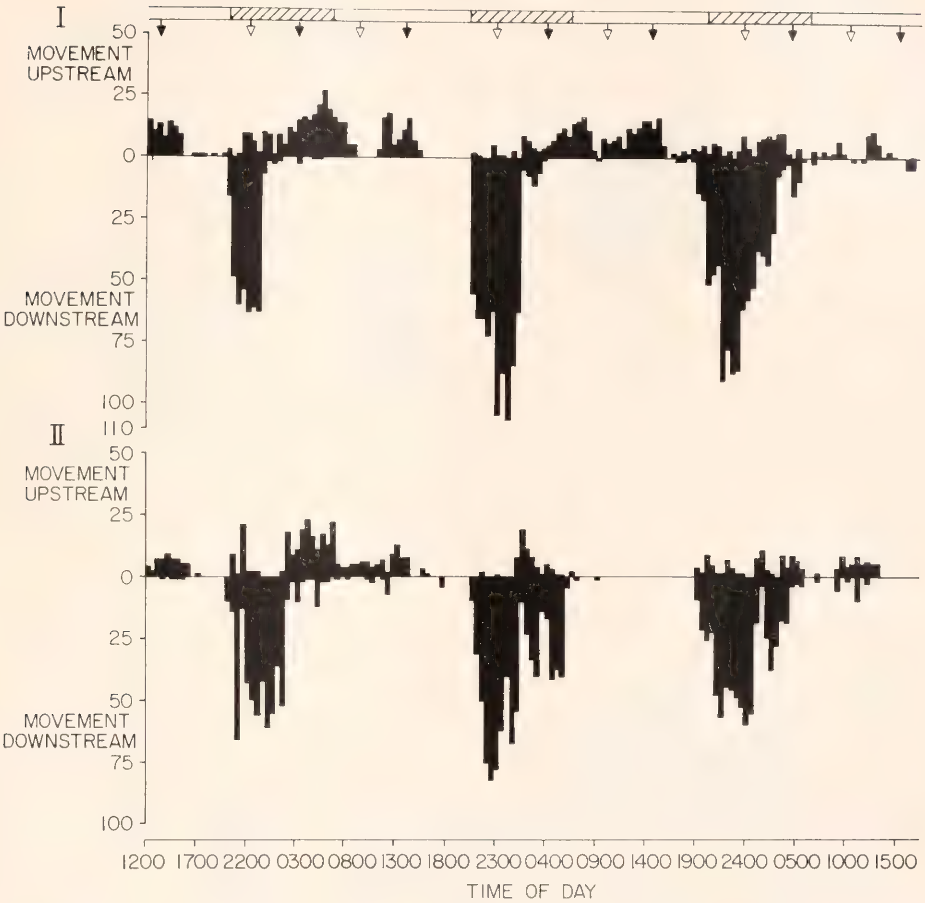

FIGURE 2. The pattern of swimming of juvenile shrimp collected from an early evening ebb tide (Full Moon). The shrimp were maintained under constant conditions within two current chambers (I and II) for three days following their collection from nature. Swimming was recorded in terms of the number of individuals swimming upstream and downstream during two minute observation periods made at 30 minute intervals. For further explanation see Figure 1.

In shrimp collected both from early and late ebb tides it appeared that upstream swimming, at what may be considered appropriate times, is only possible in recently collected animals. Those maintained in a current chamber for longer than a day or two often appear incapable of the "effort" involved and will frequently drift or swim downstream.

DISCUSSION

Postlarvae

An endogenous tidal rhythm of swimming was shown in which upstream and downstream swimming were manifested at the times of flood and ebb tides,

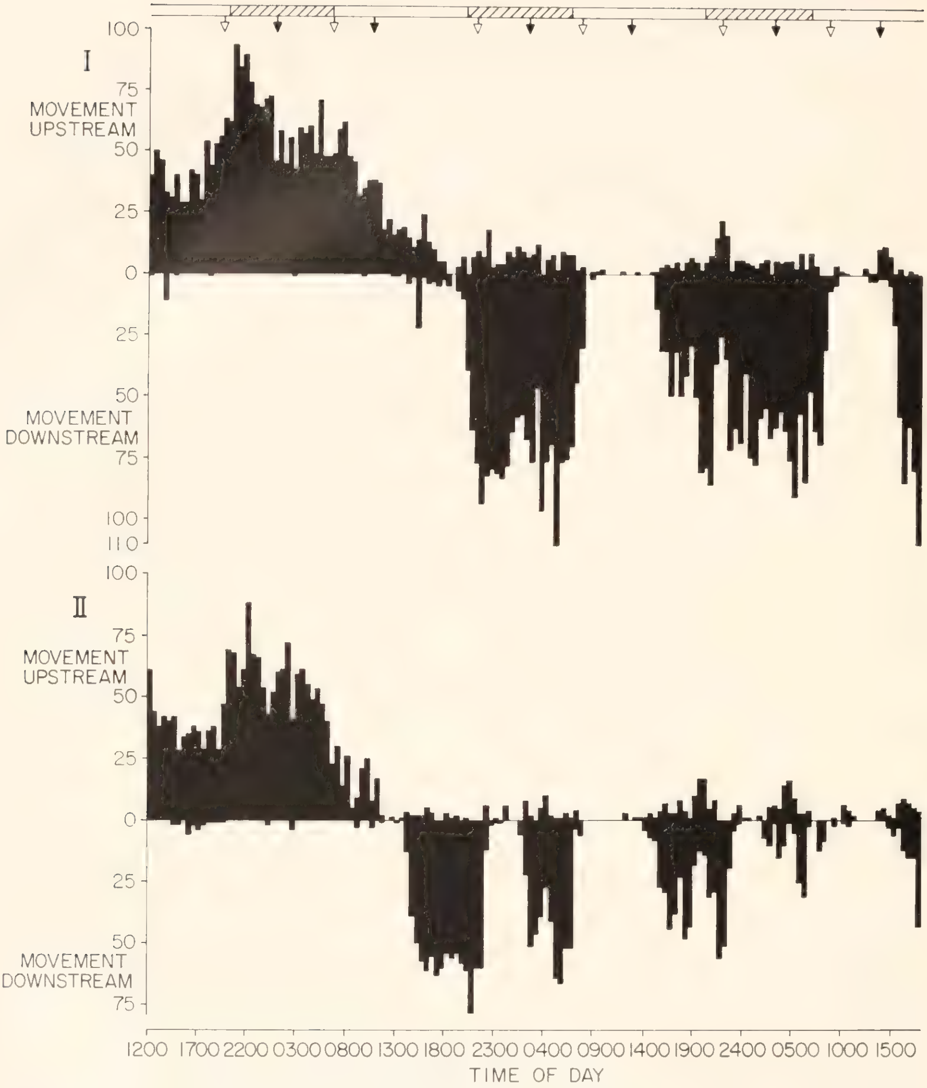

FIGURE 3. The pattern of swimming of juvenile shrimp collected from a late night ebb tide (Quarter Moon). For further explanation see Figure 1.

respectively, in the complete absence of externally changing environmental factors. The upstream and downstream phases which predominated at the times of alternate tides are not only phases during which shrimp swim in opposite directions with respect to the current but also differ with respect to the level of activity involved. The level of activity involved in downstream swimming, is considerably lower than that employed when swimming upstream. This is evident from the fact that counts, made during times of downstream swimming, were approximately of the same magnitude as those made during times of upstream swimming, whereas with the aid of the current, shrimp, actively swimming downstream, would be able to cover a far greater distance in unit time than those swimming against the same current.

The precise mechanism whereby these periods of up and downstream swimming, in phase with alternate tides, enable the postlarvae to utilize the tides for their displacements is not clear, but it is probable that during the active flood tide period the animals penetrate the water column above the substrate. In this position they are readily displaced in an inshore direction by the current. During the relatively inactive ebb tide period their movements may be confined to the substrate where, despite their downstream orientation, displacement by the current is minimal. This interpretation is in accord with the hypothesis previously expressed (Hughes, 1969a) to account for the mechanism whereby responses to salinity changes effect tide-associated displacements of postlarvae. Here it was thought that the increase in salinity, correlated with the flood tide, elicted activity within the water column, whereas the decrease of salinity at the time of ebb tide depressed activity and, with the marked reluctance of shrimp to move from water of higher to water of lower salinity, confined them to the substrate where they would more readily evade displacement by the current.

It is also evident that there is no essential difference between the orientation of postlarvae and juveniles with respect to currents at times of ebb and flood tide. Both appear to swim against flood tides and with ebb tides. The fact that they are displaced in opposite directions by the tides is explicable in terms of other aspects of their behavior which determines their presence or absence in the water column. High activity of postlarvae at times of flood tide leads them into the water column where they are readily displaced in an inshore direction. The active juveniles, however, remain in almost constant contact with the substrate orienting into and swimming strongly against the current, thereby resisting displacement.

Juveniles

In a previous publication (Hughes, 1969b) evidence was presented which indicated that swimming of migrating juvenile pink shrimp is under some measure of endogenous control. The observations reported in that publication were not carried out for longer than two days after collection of the animals, and the shrimp were exposed in the current chamber to a day-night cycle similar to that in nature. The light-off and light-on stimuli at the time of "sunset" and "sunrise," respectively, remained as effective cues for the phasing of swimming activity. On the other hand, the experiments reported here were run for three days under constant conditions in which the animals could, presumably, obtain no cues from their environment.

Although the results of the earlier experiments were confirmed and it has been clearly shown that swimming is under endogenous control there is nevertheless much about the form of the patterns obtained which is not clear. The pattern of swimming of shrimp collected at times of new and full moon (Fig. 2) still differed markedly from that of shrimp collected at the time of the quarter moons (Fig. 3) and only in the case of shrimp collected from ebb tides occurring early in the evening (full and new moons), is the adaptive phasing of the pattern of swimming with the natural tidal and diurnal cycle seemingly apparent.

The actual patterns obtained in the current chambers are probably a consequence of the interactions of oscillations of the tidal and circadian timing mechanisms. It may be that in the absence of appropriate environmental stimuli, the interaction of the two oscillating systems, tidal and circadian, gives rise to patterns of swimming activity which reflect the relative strengths of various Zeitgeber associated with the two systems. For example, in the case of shrimp collected from a late evening ebb tide (Fig. 3) it appears that periods of swimming were terminated at the time of dawn but were initiated, after the first night, at the time of the start of an afternoon ebb tide.

It is not evident why downstream swimming is manifested in the current chamber only by juveniles collected from ebb tides occurring in the early evening and, even then, only during the time of early evening ebb tides. Presumably endogenously induced downstream swimming at all night-time ebb tides would be more advantageous, and certainly more comprehensible in terms of the underlying rhythm involved.

Circadian rhythmicity in migrating postlarvae

It is well established that migrating postlarval pink shrimp may be collected from within the water column almost exclusively during the hours of darkness (Tabb *et al.*, 1962; Williams and Deubler, 1968; Roessler and Rehrer, in press). This relationship results apparently from a direct behavioral response of the postlarvae to light and there is, as indicated by these results, no endogenous component serving to confine activity to night. This contention is supported by the above mentioned workers who all record circumstances in which the behavior of postlarvae were influenced by prevailing light intensities. Tabb and his co-workers found that almost as many postlarvae were caught on a flood tide in the afternoon of a cloudy day as were caught after dark on the same day. Williams and Deubler on the other hand, found that a bright light at night, in the vicinity of their collecting station, drastically reduced their catch. In addition they found that higher catches were made at times of new moon than full moon. This was substantiated by Roessler and Rehrer who found that the postlarvae were closer to the substrate at full moon but more evenly distributed in the water column at times of new moon.

Circadian rhythmicity in migrating juveniles

These results are in accord with those of an earlier study (Hughes, 1968) in which it was found that the diurnal pattern of activity of juvenile shrimp greater than about 4 cm in total length was under a considerable measure of endogenous control, whereas shrimp smaller than this size exhibited an apparently greater responsiveness to exogenous stimuli and a lesser dependence on the endogenous rhythm. Unlike the results of this earlier study the results presented here do not show clear persistence of the diurnal pattern of activity and inactivity at night and by day respectively. It seems that the influence of the tidal cycle is manifest only when the shrimp are maintained with a current of water and not, as in the previous experiments, when maintained in aquarium tanks. In these experiments the interaction of this tidal rhythm with the circadian apparently obscures the clear manifestation of either. However, the circadian rhythm, which undoubtedly contributes to maintaining the diurnal periodicity of activity, is evident. Its influence is exhibited by virtue of the fact that times of inactivity occur only during the time of day. Circadian rhythmicity is further evident in the case of the juveniles collected from an early evening ebb tide (Fig. 2) the periods of downstream swimming are initiated each day at the time of sunset while in the case of those collected from late night ebb tides (Fig. 3) downstream swimming is terminated each day at the time of sunrise.

Adaptive significance of endogenous control

The efficacy of tide-associated displacements as a mechanism to enable movements into or out of inshore waters is probably greatly enhanced by the endogenous phasing of adaptively appropriate behavior with the prevailing tide cycle.

Direct response to the water quality (or other factors indicating change of tide) would not, for several reasons, be as effective a mechanism as one in which this direct response is further controlled endogenously. The most obvious problem overcome through endogenous control is one which was raised by Creutzberg (1961) in his study of tide-associated displacements of elvers. He found it difficult to explain the mechanism whereby elvers which, during the flood tide, had risen to higher levels within the water column, would be able to perceive the end of the flood if they remained in that same water mass. This is a problem which would similarly face pink shrimp and all other organisms utilizing tides for their displacements. These results suggest that, in pink shrimp postlarvae the duration of the active and inactive periods coinciding with flood and ebb tides respectively is endogenously limited, affording them a degree of independence from water quality cues. The latter will still serve to maintain the phase relationship between times of activity/inactivity and the flood/ebb cycle.

In the case of the juveniles there is less evidence from these results that the endogenous tidal rhythm manifests itself overtly in adaptively advantageous behavior. The downstream swimming evidenced at the time of an early evening ebb tide by individuals collected the previous night from an early ebb tide (Fig. 2) and its subsequent reversal to upstream swimming at about the time of change of tide, is the only clear suggestion of an adaptive association existing between tides and behavior. It is probable, however, that a changing responsiveness to decreasing or increasing salinity, or whatever other stimulus denotes tidal change, exists and that at times of change of tide the animal is maximally responsive to the appropriate stimulus and maximally "geared" to carry out appropriately adaptive behavior for an interval equivalent to the duration of the tide.

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SUMMARY

1. Swimming of both postlarval and juvenile pink shrimp was recorded in current chambers in the laboratory for three days following collection from nature.

2. In the apparent absence of environmental cues the animals maintained various forms of phase relationship with the tidal and diurnal cycles.

3. Postlarvae manifested a pattern of swimming, markedly in phase with the semi-diurnal tide cycle. Upstream swimming took place during flood tides and downstream swimming during ebb tides. No circadian periodicity was found and the confining of their activity in nature to night-time is considered a direct response to prevailing light intensity.

4. The patterns of swimming evidenced by juveniles differ depending, apparently, on some as yet undetermined aspect of the tide cycle to which they are exposed prior to collection. Individuals collected at times of new and full moon, when ebb tides occur early in the evening, exhibit a different pattern of swimming from those of individuals collected at times of quarter moons when ebb tides occur late at night. The patterns obtained are clearly endogenous although their adaptive phasing with the tidal and diurnal cycles is not always evident.

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INJURY INDUCED MOLTING IN *GALLERIA MELLONELLA* LARVAE

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Injury to larval or nymphal insects usually causes a delay in molting (O'Farrell and Stock, 1953; Pohley, 1959, 1960). When an appendage of a cockroach is amputated (O'Farrell, Stock and Morgan, 1956) or an imaginal wing disc is extirpated from lepidopteran larvae (*e.g., Ephestia*, Pohley, 1960; *Galleria*, Madhavan and Schneiderman, 1969) there is a delay in the onset of the succeeding molt. This delay is particularly obvious when the injury is inflicted before preparations for the succeeding molt are initiated and appears to be proportional to the magnitude of injury. Based on these facts, injury induced delay of molting is attributed to the regenerating tissues (see Pohley, 1967; Bodenstein, 1959; and Madhavan and Schneiderman, 1969) which somehow inhibit the normal initiation of molt. As insect molt is controlled by brain and ecdysial glands (*cf.*, Novak, 1966), the injury may cause delay in molting by inhibiting either brain or prothoracic glands, but the actual mechanism of injury induced delay of molting is not clear. In contrast to this, injury to chilled, debrained, diapausing pupae of saturniid moth, *Hyalophora*, activities the prothoracic glands to secrete molting hormone and initiate adult development (McDaniel and Berry, 1967).

We examined the effect of injury on molting in last larval (7th) instar of *Galleria mellonella* and present evidence that early (less than 24 hr old) last instar larvae undergo an extra larval molt within 96 hours after injury, while mid instar larvae (4 day old) delay their molting. The results also suggest that injury induction of molting in early last instar larvae is mediated via the brain which appears to activate prothoracic glands and possibly the corpora allata. Furthermore, unlike during a normal larval molt, only a small percentage of the epidermal cells of larvae induced to undergo an extra larval molt, engage in DNA synthesis.

MATERIALS AND METHODS

Galleria mellonella larvae are reared in the laboratory at 29° C, 70% R.H. on an artifical diet consisting of mixed cereal (Gerber Baby Foods), glycerine, honey and bee's wax in the proportion 12:1:1:0·5 by volume. In the strain of *Galleria* used in this study, freshly molted last instar larvae are colorless but become pigmented a few hours after molt. They can also be recognized by the size of the head capsule and body weight (Beck, 1970). Accurately timed larvae are separated from the culture and maintained in plastic petri dishes with food. These larvae normally pupate in about 8 to 9 days and in our laboratory do not normally undergo an additional larval molt unless injured.

Unless otherwise specified, the term larva refers to 7th (last) instar larvae which were used in all the experiments reported here. The terms early, mid and late instar refer to less than 1, 4 and 6-day-old last instar larvae, respectively. Six-day-old larvae spin a coccoon on isolation but have not entered pharate pupal stage.

The larvae were routinely anaesthetized by placing them on crushed ice and the actual surgical maneuvers were also carried out while the larvae were on ice. Other modes of anesthesia applied in these studies were the use of CO_2 and etherization. The standard injury was to remove the right metathoracic wing disc. A mixture of phenylthiourea, streptomycin and penicillin (2:1:1) was placed in the wound to prevent infection and quinone formation and the wound was sealed by a blood clot formed during a 30 minute period on ice. In addition to the standard injury, transection of the ventral nerve cord between the 5th and 6th abdominal segments and integumental injuries were also used in some experiments. The integumental injury consisted of an operation to cut open the integument as if it were to remove the wing disc. The surgical procedures were similar to those described by Schneiderman (1967; see also Madhavan and Schneiderman, 1969). Each of the experiments was repeated at least twice with essentially similar results. The data presented are those of a typical experiment.

Table I

Effect of injury on molting in last instar larvae of Galleria mellonella

Age of larva in days	Treatment	Number of larvae	No. molted into larvae	No. of adults with regenerated wing discs
0 (0–6 hr)	Uninjured	15	0	—
0 (0–6 hr)	Wing disc removed	15	14*	13
1 (15–24 hr)	Uninjured	12	0	—
1 (15–24 hr)	Wing disc removed	25	24*	24
1 (15–24 hr)	Integumental injury	15	12	—
1 (15–24 hr)	Transection of ventral nerve cord	12	10**	—
4	Wing disc removed	18	0	15
6	Wing disc removed	12	0	0

* Some of these larvae underwent one more larval molt before pupation.

** Several of these larvae died after the larval molt. Four larvae, that survived, did not pupate in 3 weeks.

Ecdysones used in this study were inokosterone and ecdysterone (Syn. β ecdysone) purchased from Rohto Pharmaceuticals, Osaka, Japan or obtained from Mann Research Corp., Rahway, New Jersey. Ecdysones were dissolved in 10% ethanolic insect ringer to a concentration of 5 $\mu g/\mu l$. Juvenile hormone (Roeller compound) was generously provided by Hoffmann La Roche, Inc., Nutley, New Jersey. This compound was a mixture of the *cis. trans* isomers and was about 30% as active as the pure hormone obtained from Prof. A. S. Meyer, of Case Western Reserve University, Cleveland. The hormone was dissolved in peanut oil. Tritiated thymidine (H^3TdR) was purchased from New England Nuclear Corp. The specific activity was 2.0 C/mM and was dissolved in sterile distilled water to a concentration of 1 $\mu c/\mu l$. Autoradiographic procedures were similar to those reported earlier (Krishnakumaran, Berry, Oberlander and Schneiderman, 1967).

RESULTS

(1) *Injury induction of an extra larval molt*

In the first series of experiments, last instar larvae of known ages, varying from immediately after molt (0 day), 15–24 hrs (1 day), 4-day and 6-day-old, were anesthetized on ice, injured and examined for the induction of an extra larval molt or a delay in molting. The injury consisted of either removal of the right methathoracic wing disc, transection of ventral nerve cord, or an integumental injury. Uninjured controls pupated 8–9 days after the last larval molt. The results reported in Table I show that, irrespective of the type of injury, about 90% of the 0 day and 1 day old injured larvae molted into larvae within 4 days after injury. Occasionally these larvae underwent an additional larval molt before pupation. Injury to four-day-old larvae invariably caused a delay in the succeeding molt, which was a pupal molt, by about 6 to 8 days. Six-day-old last instar larvae also delayed pupation on injury but not to the same extent as the four day old injured larvae. The above results were obtained with larvae that were anesthetized on ice, however, when larvae were anesthetized under CO_2 or ether only 10 to 20% of the 0 and 1-day-old injured larvae underwent an extra larval molt. The larvae that did not undergo a molt showed a delay in pupation.

The injured larvae (in which metathoracic wing disc was extirpated) were allowed to complete adult development and the resulting adults were examined for the presence or absence of right metathoracic wing. The results show (Table I) that larvae from which wing discs were extirpated on day 1 or 4 of the stadium regenerated the wing (90%) while none of the larvae from which wing discs were removed on day 6 regenerated the wing. In one experiment, 10 larvae that completed the injury induced larval molt were dissected to determine whether they regenerated the wing disc during this molt. These larvae showed an accumulation of a tracheal mass at the site of injury but no regenerated wing disc.

(2) *Effect of juvenile hormone on injury induction of larval molt*

The titer of juvenile hormone in *Galleria* is high in the early part of the instar and is absent in older larvae (unpublished observations; *cf.*, Novak, 1966). Since this may account for the difference in the response of larvae to injury at different ages, we investigated the effect of juvenile hormone on injury induced larval molts. Injection of 2 or 4 μg of j.h. into 4 day old or 8 μg of j.h. into 6-day-old larvae did not induce a larval molt (such apparently high doses of juvenile hormone did not induce larval molts because the compound was a *cis. trans* mixture with only about 30% activity as the pure hormone *cf.*, Sehnal and Meyer, 1968). Following the application of these doses of j.h., the metathoracic wing disc was extirpated. Controls received an equal amount of peanut oil before injury. The results reported in Table II show that the presence of juvenile hormone at the time of injury did not influence the induction of molt, and regeneration of wing discs.

(3) *Effect of injury on juvenile hormone induced super larvae*

Early last instar larvae, unlike older larvae, respond to injury by initiating a larval molt. A larvae that molted once in response to injury undergoes an additional molt if injured again soon after the molt. Is this ability to respond to injury limited to injury to natural 7th instar larvae only; or do 3 and 4-day-old last instar larvae induced to undergo an additional larval molt by injection of an adequate dose of juvenile hormone (Sehnal and Meyer, 1968) respond to injury by undergoing an additional larval molt. To determine this, several 3 to 4-day-old last instar larvae were injected with 10 μg/larva juvenile hormone: a dose adequate to cause perfect additional larval molts in 4 to 8 days. J.h. induced super larvae when left untreated, continued to feed, completed normal larval development and pupated 9 to 12 days later. Ten of these larvae were injured, by removal of a wing disc, on the day of molt while another ten were maintained as untreated controls. These larvae were examined to determine the time and nature of the succeeding molt. Both controls and experimental animals pupated in about 10 to 12 days without an additional larval molt. Furthermore, these larvae did not regenerate the wing disc before pupation. These results show that j.h. induced super larvae differ from normal last instar larvae in their response to injury.

(4) *Role of brain in induction of supernumerary molt*

Is the injury induction of larval molt in early 7th instar *Galleria* larvae the result of activation of brain or ecdysial glands? To determine this, 60 one day old larvae were injured and immediately after injury, 24, 48, or 72 hrs later, they were ligated behind the brain. Uninjured 1, 2, 3, and 4 day old larvae were ligated and served as controls. These preparations were maintained for 15 days and examined for deposition of a new cuticle. All 50 control larvae and all larvae ligated within 48 hr after injury failed to deposit a new cuticle. About 20% of larvae that were ligated 72 hr after injury deposited a new cuticle. These results show that the brain is essential for injury induction of a supernumerary molt. Furthermore, these data also suggest that injury does not induce the molt either by activating the prothoracic glands or by directly influencing the chitogenous epithelium.

TABLE II

Effect of juvenile hormone on injury induced larval molt

Age in days	Amount of j.h. injected (μg)	No. of larvae	No. undergoing additional larval molt	No. undergoing pupal molt	No. of adults regenerating wing discs
1	4	15	9*	9	9
	control	15	12*	12	12
4	2	15	0	11*	9
	4	15	0	9*	9
	control	15	0	10*	—
6	4	12	0	9**	0
	8	12	0	8**	0
	control	12	0	10	0

Controls received an equal volume of peanut oil.
* Remaining larvae died.
** The others died before pupation. Usually these delayed pupal molt by 7 to 10 days.

Direct evidence for the role of the brain in the induction of an extra larval molt was obtained by implantation of brains from injured early larvae into older injured larvae. Several one day old last instar larvae were injured. One or two days later, their brains were carefully dissected out and cleaned of all attached glands and nerves and implanted into 4 and 6-day-old larvae. Routinely two brains were implanted into each 6-day-old larva, while 4-day-old larvae received only one brain. In another experiment the head capsule of an injured larva containing the brain, its associated glands, and subesophageal ganglion, was implanted into 4-day-old larvae. Controls received brains or head capsules from uninjured 2 or 3-day-old larvae. These were examined for the induction of molt and if they deposited a cuticle the nature of molt was also determined. There were no differences between the larvae that received a brain alone and those that received the head capsule with brain and the associated neuroendocrine glands.

Results are reported in Table III, and show that the brain from uninjured 2 to 3-day-old larvae (unlike those of injured larvae) did not induce a precocious molt, confirming the earlier observations of Beck (1970). In one limited experiment (with 4 larvae) 3 brains from uninjured early larvae were implanted into each larva, but even these failed to induce a prompt molt whether larval or pupal. However, over 70% of the 4 day old larvae that received a brain from injured larvae underwent an extra larval molt within 4 to 6 days. Such super larvae subsequently pupated and developed into apparently normal adults. Although the implanted brain usually induced an extra larval molt, occasionally a larva pupated promptly in response to the implanted brain. A small number of 6-day-old larvae also molted into larvae 7 days after implantation of 2 activated brains. One of these larvae had a larval body with everted imaginal wing discs and pupal tanning in the middorsal line of the thoracic and anterior abdominal segments, and was capable of larval-type movements. Similar larval-pupal intermediates were obtained by injection of j.h. in 6-day-old *Galleria* larvae by Sehnal and Meyer (1968; Krishnakumaran, unpublished observations).

TABLE III

Effect of implantation of injured early larval brain on molting in 4 and 6 day old larvae

Stage and treatment		No. of brains implanted	No. of recipient larvae	No. undergoing a prompt molt
Donor	Host			
3-day-old normal larva	4-day-old intact larva	1	12	0
2–3-day-old injured larva	4-day-old intact larva	1	25	17*
3-day-old normal larva	6-day-old intact larva	2	10	0
2–3-day-old injured larva	6-day-old intact larva	2	22	4*
3-day-old normal larva	4-day-old larva ligated behind head	2	12	1**
2–3-day old injured larva	4-day-old larva ligated behind head	2	12	7**
—	4-day-old larva ligated behind head	0	12	0

* The additional molt was induced in 4 to 6 days and was usually larval. One larva molted into a pupa.

** This molt was pupal except in two cases and occurred between 11 and 16 days after implantation of brains.

TABLE IV

Pattern of DNA synthesis in various tissues during normal and injury induced larval molts in Galleria

Tissue age in days	6th Instar	7th Instar							
	Normal larval-larval molt 1–5*	Uninjured larvae			Injury induced molt				
		1	2	3	1	2	3	4	1–4**
Epidermis	++++	0	0	+	0	0	+	+	+
Trachea	++	0	0	—	0	0	+	+	+
Midgut	+++	+	+	+	+	+	+	+	+
Fat body	++++	+	+	+	+	+	+	+	+
Muscle	+++++	+	+	+	+	+	+	+	+
Hemocytes	++	+	+	+	+	+	+	+	+
Malpighian tubules	+++++	+	+	+	+	+	+	+	+

+ Incorporated H^3TdR. Each + represents up to 15% of cells examined.
0 No incorporation of H^3TdR. — Data not available.
Larvae were injected with 3 μc/larva H^3TdR and sacrificed 24 hr later.
Four larvae were examined on each day.
* Larvae were injected with 1 μc/larva H^3TdR on each of the five days of the 6th stadium.
** Larvae were injected with 3 μc/larva H^3TdR at the time of and, 48 and 72 hr after injury.

Further evidence to show that brains from early 7th instar larvae are capable of inducing a molt was obtained by implantation of the brains into ligated larval abdomens. Four day old *Galleria* larvae when ligated behind the head capsule so as to deprive them of a source of brain hormone, survive for several weeks but do not molt. Twelve of these ligated larval abdomens received two brains each from injured early larvae. Controls either received brains from 2 to 3-day-old uninjured larvae or were untreated. Between 11 and 16 days after implantation of the brains, 56% of the larvae that received the injured larval brain and 8% of the controls molted (Table III) while none of the untreated controls molted even after 4 weeks. The molt induced by the implanted brain was usually a pupal molt although, in one experiment, two larval abdomens deposited a distinctly larval cuticle with well developed prolegs, larval bristles and uneverted wing and leg imaginal discs. Possibly in these two preparations allata were implanted along with the brain.

These results show that brains from injured, unlike those of uninjured early 7th stadium *Galleria* larvae are capable of activating the ecdysial glands.

(5) *Injury induced delay in molting*

Mid (4 day) and late (6 day) last instar larvae when injured, delay subsequent pupation. This delay in molt could be the result of inability of (a) the chitogenous epithelia to respond to ecdysones, (b) the ecdysial glands to brains hormones, or (c) the failure of the brain to activate the ecdysial glands. The fact that active brains (from injured early larvae) initiated a molt in injured 4-day-old larvae suggested that the ecdysial glands are not refractile. Moreover, these results also show that the epidermal cells of injured mid larvae are not unresponsive to ecdysones. This conclusion was further supported by injection of exogenous ecdysones. Twelve injured 4 day old larvae were injected with 10 μg/larva ecdysterone soon after injury, and control larvae received Ringers' fluid. While all controls pupated in 8 to 10 days, all ecdysterone treated larvae deposited a new cuticle within 72 hours after injection, showing that the epidermal cells of these injured larvae can respond to ecdysone. Further, it is interesting to note that ecdysterone treated larvae secreted a larval-pupal intermediate type of cuticle. Thus, it was concluded that the injury induced delay in these larvae is not due to inability of chitogenous epithelium to respond to ecdysone and that the delay may be mediated via the brain.

The next question we asked was, whether the inactivity of the brain in injured 4-day-old larvae was the result of absence of stimulus or the presence of a molt inhibitory agent. To determine this, two brains from injured 4-day-old larvae were implanted into each injured 1-day-old larva. If a molt inhibitory factor was present in these brains, it may be expected to suppress or delay the injury induced molt in the host larvae. All ten larvae that received these brains, molted about the same time as the controls, suggesting that injury induced delay of molting in mid larvae was not the result of the presence of a molt inhibitory hormone but may be due to the absence of the normal stimulus that activates the brain.

(6) *DNA synthesis during normal and injury induced larval molts*

In the course of normal larval molt, epidermal cells in a variety of insects engage in extensive DNA synthesis (Bowers and Williams, 1964; Krishnakumaran *et al.*, 1967). What are the patterns of DNA synthesis in the various tissues during a normal and injury induced molts in *Galleria*? We examined this by determining, autoradiographically, the incorporation of H^3TdR in 6th stadium larvae (penultimate larval instar) and during an injury induced molt in 7th instar larvae.

Six larvae were isolated soon after their molt into 6th stadium. These received 1 μc/larva H^3TdR on each of 5 days of this stadium, and were killed soon after molting into 7th stadium larvae. Autoradiographic analysis of the pattern of incorporation of H^3TdR in different tissues of these larvae showed that about 70% of the epidermal cells, 80% of the muscle cells and malpighian tubule cells and 60% of the fat body cells incorporated H^3TdR into DNA of their nuclei (see Table IV). These results are comparable to the observations made on larval *Cynthia ricini* (Krishnakumaran *et al.*, 1967).

The pattern of DNA synthesis during an injury induced larval molt was examined next. For this purpose 16 newly molted 7th stadium larvae were injured. Immediately after injury (0 day), 1, 2, or 3 days after injury, these larvae were injected with 3 μc/larva H^3TdR. These larvae were sacrificed 24 hr after injection of isotope. Another batch of 4 injured larvae was injected with 3 μc/larva H^3TdR immediately after, 48 and 72 hr after injury. These larvae were killed after the subsequent molt and autoradiographs of tissues away from the site of injury were prepared. Uninjured larvae served as "controls." Autoradiographic data regarding the numbers of nuclei that incorporated thymidine are presented in Table IV and indicate that during the first 48 hr of the last instar there was no incorporation of H^3TdR in the epidermal cells and even in the other tissues, DNA synthesis was not extensive. In larvae that received isotope between 48 and 72 hr 10–15%

of epidermal cells engaged in DNA synthesis. During the first 48 hr of an injury induced molt only about 5% of the cells in muscle, fat body, malpighian tubules and other tissues incorporated tritiated thymidine. As in uninjured controls, no labelled epidermal cells were observed in larvae during the first 48 hours after injury. Even in larvae that received the isotope 48 or 72 hours after injury only 4 to 5% of the epidermal cells, fat body cells, hemocytes, muscle cells and malpighian tubule cells engage in DNA synthesis. Thus three day old uninjured "controls" show a higher rate of incorporation of H^3TdR than the corresponding injured larvae. Even in larvae that were exposed to isotope throughout the entire period of injury induced molt, only about 10% of the fat body, muscle, epidermal, malpighian tubule and pericardial cells incorporated label. Thus, unlike in the normal larval-larval molt (during a penultimate larval stadium), DNA synthesis in cells of larvae during an injury induced molt was much more restricted.

Discussion

Role of the brain in the injury induction of molt

The results reported in the present account clearly show that injury to last instar larvae within 24 hr after molt, leads to an additional larval molt. However, older larvae do not respond in the same manner. The results also show that the brain is involved in injury induced molting in *Galleria* larvae. The fact that larvae ligated behind the brain as late as 48 hr after injury fail to molt, and that brains from injured early last instar larvae induce molting in intact mid and late last instar larvae as well as larvae ligated behind the head capsule, support this conclusion. As brains from uninjured 7th stadium larvae of corresponding chronological age do not activate ecdysial glands and induce a molt (a finding that confirms the earlier observations of Beck (1970) in these larvae), and the fact that the brain from injured larva is capable of initiating a molt suggests that injury somehow activates the brain. The mode of activation of the brain in lepidopteran larvae is obscure, but may involve the nervous system as was suggested by Edwards (1966). This conclusion is supported by the experiments using bracon venom (a potent inhibitor of neural activity), (Edwards and Sernka, 1969), physical restraint (Edwards, 1966) and nerve transection (Sehnal and Edwards, 1969) all of which inhibited normal metamorphosis of *Galleria* larvae. The present observations on the nature of response to injury under different anaesthetic agents also support such a conclusion. While larvae anesthetized on ice and injured underwent a prompt larval molt, larvae anesthetized under CO_2 or ether failed to respond in a similar way. These three anesthetics are known to affect nerve conduction in invertebrates differently (Caldwell, 1958; Hafemann, 1969). Carbon dioxide anesthesia results in an irreversible loss of ability to transmit nerve impulses, while cold does not cause such an irreversible change in invertebrate nerves (Hafemann, 1969). Preliminary studies on the isolated ventral nerve cord from *Galleria* 7th stadium larvae showed similar differences between CO_2 and cold anesthesia. Moreover, etherization and nitrous oxide also cause irreversible damage to the nerves (Hafemann and Krishnakumaran, unpublished observations). (The fact that Pohley (1960) and Madhavan and Schneidermann (1969) did not observe in *Ephestia* and *Galleria* larvae any injury induced larval molts may be attributed to two factors. First they used older larvae and secondly they used ether for anesthetizing the larvae.) Thus, it is suggested that injury to early last instar larvae somehow mimics the normal stimulus that activates the brain and that this stimulus may be neural. Consequent to this, the early last stadium larvae undergoes molt on injury.

Although the early 7th stadium larva responds to injury by a prompt molt, injury to 4-day-old larvae causes a delay in the subsequent molt. The fact that injured larvae respond to exogenous ecdysones, by depositing a cuticle, suggests that the epidermal cells are not inhibited by the regenerating tissues. This finding confirms similar observations by Madhavan and Schneiderman (1969). In addition, the fact that implantation of an activated brain into such injured larvae induced a prompt molt, indicates that the prothoracic glands are not inhibited by the injury or the regenerating tissues. All these suggest that the delaying effect of injury to mid last instar larvae must also be mediated via the brain. The fact that implantation of brains from injured 4-day-old larvae into injured early last instar larvae did not cause a delay in initiation of molting suggests that the injury induced delay does not involve a molt inhibiting hormone but is possibly the result of the absence of the stimulus to activate the brain. However, it is not clear why the early and mid last instar larvae behave differently in response to injury.

Activation of allata

Injury to early last instar larvae caused an additional larval molt in about 4 days, which may be the result of the presence of juvenile hormone in the larvae at the time of initiation of this molt. In 24 and 48-hour-old last instar *Galleria* larvae extractable juvenile hormone titers are present and hence injury induction of larval molting is not surprising. However, implantation of brains from injured early larvae into 4 or even 6-day-old larvae (at which stages there is no extractable juvenile hormone) also induced larval molts. There are several indications that 4 to 6-day-old larval epidermis will deposit larval cuticle only if there is sufficiently high titer of juvenile hormones. For example on injection of beta ecdysone in 4-day-old larvae they deposit a larval-pupal intermediate type cuticle while 6-day-old larvae deposit a pupal cuticle suggesting that the epidermal cells at this stage secrete a pupal cuticle if forced to deposit a cuticle without any delay (*cf.*, Krishnakumaran, Granger and Schneiderman, unpublished observations). Sehnal and Meyer (1968) reported that 4 to 6-day-old 7th instar larvae deposit a larval cuticle only on application of large doses of juvenile hormone suggesting that normally 4 to 6-day-old 7th stadium *Galleria* larvae are committed for synthesis of pupal cuticle and that only in the presence of sufficient quantity of juvenile hormone do these larvae reprogram to deposit a larval cuticle.

How does a brain from an injured early 7th instar larva induce a larval molt in 4 and 6-day-old larvae? A likely explanation is that the implanted brain activated allata to secret juvenile hormone. The fact that implantation of a brain, devoid of all attached connectives and glands, induced larval molts, suggests that the implanted brain can activate the host corpora allata as well as the ecdysial glands. This view is further supported by the observation that 4-day-old larvae ligated behind the head usually pupate in response to implantation of an injury activated brain. (Ligation behind the head capsule removes brain and also the allata but retains the ecdysial glands.) From these observations it is concluded that injury activated brains possess not only prothoracotropic but also allatotropic activity. Injury activation of allata is not uncommon in insects. In *Leucophaea maderae* Luscher and Engelmann (1960) reported that injury caused a continued activity of allata and supernumerary molts. Similarly amputation of antennae in *Periplaneta americana* early in a stadium reduced the rate of metamorphosis (Pohley, 1962). Whether the allatal activity in these two insects is regulated by a blood borne factor or by a nerve stimulus is not certain. The fact that in *Leucophaea*, transection of allatal nerves did not affect the induction of supernumerary nymphal molts suggests that the injury induced allatal activity does not require neural stimuli. In the earwig, *Anisolabis*, Ozeki (1962) reported injury stimulation of allata. However, in this insect nerve stimuli were shown to be necessary for this activation. The allatotropic factor in *Galleria* larvae may be a blood borne factor and is presumably a neurosecretory product as in some orthopterans (*Schistocerca*, Strong, 1965; *Locusta*, Girardie, 1966).

DNA synthesis and molting

The results reported here show that larval epidermal cells during the course of an injury induced larval molt are not actively engaged in DNA synthesis unlike during a normal larval-larval molt (penultimate larval instar of *Galleria* and 4th instar larvae of *Cynthia ricini*, Kirshnakumaran *et al.*, 1967). The number of cells that were engaged in DNA synthesis during an injury induced molt appears comparable to the number that normally incorporate H^3TdR during the first two days after the last larval molt. However, in 3-day-old last instar larvae, 10 to 15% of the epidermal cells engage in DNA synthesis which is more than in the injury induced molting larvae of corresponding chronological age. Thus it is clear that DNA synthesis during injury induced larval molt is not as extensive as in the penultimate instar larvae undergoing a normal larval molt, and that very few epidermal cells in these larvae replicate their DNA prior to molting. This condition reminds one of the pattern of DNA synthesis in the epidermal cells during adult-adult molts in *Cynthia* moths grafted to pupae (Krishnakumaran *et al.*, 1967), and in the ecdysterone injected *Cynthia ricini* larvae and *Tenebrio* pupae (Krishnakumaran, Granger and Schneiderman, unpublished observations).

There are several possible explanations for the relatively low level of DNA synthesis in epidermal cells of injury induced molting larvae. First, contrary to earlier suggestions, DNA synthesis normally noticed in the epidermal cells of larval insects in preparation to molting is unrelated to the presence of ecdysones (Kirshnakumaran, 1962; Bowers and Williams, 1964; Krishnakumaran *et al.*, 1967). Secondly, injury induced molt differs from the normal larval molts in the absence of an increase in size of the larva. Consequent to the lack of increase in size, there is no need for an increased number of cells and this resulted in a molt in the absence of extensive DNA synthesis in the larval cells (*cf.*, Wigglesworth, 1964). Thirdly, as injury induced the molt, even before the synthetic processes for the preceding molt were completed (*e.g.* deposition of the inner layers of the endocuticle and secretion of wax and cement layers of the epicuticle; processes which are under hormonal control, *cf.*, Locke, 1970), the levels of ecdysone may be continuously high throughout the entire injury induced molt cycle. The low level of DNA synthesis in larval cells during an injury induced molt in *Galleria* larvae may be the result of the presence of high titers of beta ecdysone which is known to inhibit DNA synthesis and promote cuticle deposition (Oberlander, 1969, 1972).

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Summary

1. Injury to less than 1-day-old last instar larvae of *Galleria* induced an additional larval molt.

2. 4 and 6-day-old larvae on injury delay their succeeding pupal molt.

3. Injury induction of an additional molt as well as the delay of molting appear to be mediated via the brain.

4. Brains from injured early last instar larvae, unlike those of the uninjured larvae, appear to possess prothoracotropic and allatotropic activities.

5. During an injury induced molt the epidermal cells do not engage in DNA synthesis to the same extent as in the normal larval molts.

6. The relevance of these results in the analysis of control of molting and DNA synthesis is discussed.

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EFFECTS OF ECDYSTERONE ON THE DEPOSITION OF COCKROACH CUTICLE *IN VITRO*

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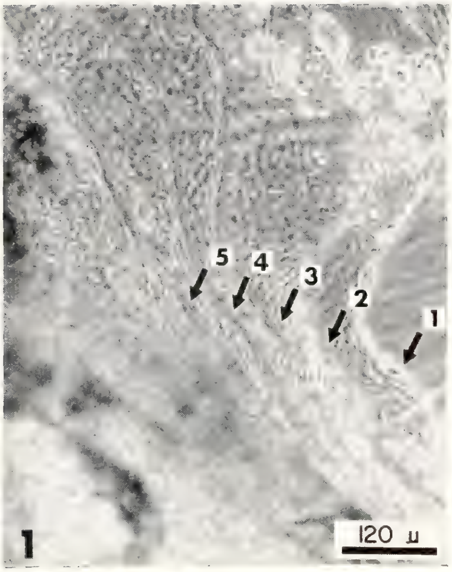
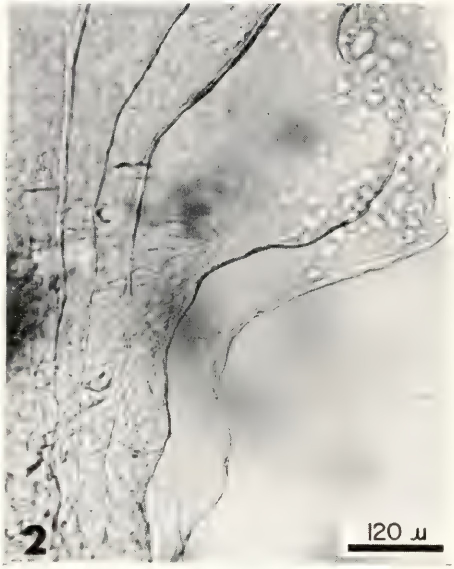
The production of cuticular materials by insect tissue *in vitro* in response to stimulation by the molting hormone was discussed in a recent review (Marks, 1970). In these early studies, relatively large amounts (3 to 25 μg/ml of medium) of the hormone were used, the period of exposure ranged from 2 to 10 days, and no systematic attempt was made to determine the effects of different doses. In a recent study, Marks and Leopold (1971) reported that cuticle deposition in cockroach leg regenerates could be induced *in vitro*. High doses (2 to 25 μg/ml) given over long periods (7 to 10 days) resulted in the deposition of a heavy cuticle complete with well-defined setae; smaller doses produced a thinner cuticle on which droplets of chitin-bearing material accumulated. The lowest concentration that induced any deposition was 0.1 μg/ml given over a period of 7 days. These results suggested that a quantitative study of hormonal induction of cuticular deposition *in vitro* might facilitate the investigation of the mechanisms whereby ecdysterone activates the epidermal cells and of the process of cuticle deposition.

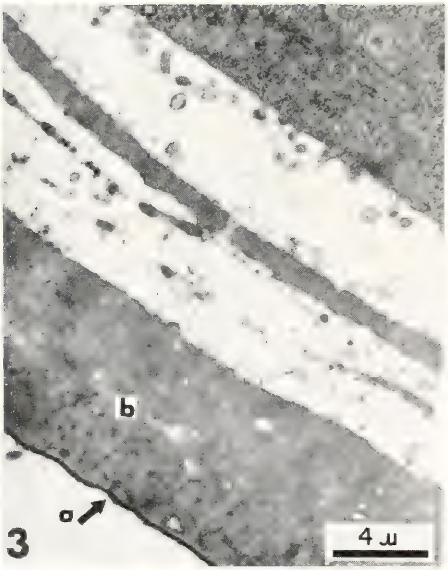
In the present study, the effects of three dose-related variables were investigated: the concentration of the hormone, the duration of exposure, and interruption of the dose.

METHODS

Late-instar nymphs of the Madeira cockroach, *Leucophaea maderae* (F.) were removed from the laboratory colony immediately after molting. The mesothoracic legs were removed 24 hr later at the femora-trochenteral joint. The operated insects were held for 24–26 days at room temperature in paper cups and fed dog food and water. At the end of this period, the coxae were dissected, and the regenerating legs, which at this stage comprised about 2 mm^3 of tissue each, were removed and placed in multipurpose tissue chambers (Rose, 1954) filled with 2 ml of M20 nutrient medium containing 5% fetal bovine serum. These were maintained at 27° C. The ecdysterone dissolved in water (1.0 or 0.1 μg/μl) was held under refrigeration, and a new solution was made up every 30 days. The hormone was injected into the chambers, and at the end of the period of exposure, the treated medium was removed and the chambers were rinsed twice and refilled with fresh medium. All chambers were observed periodically for 14 days after exposure to the hormone and then scored for the presence of cuticle. Since the thickness of the cuticle varied with the dose, a positive score was given only if both chitin droplets and a cuticular membrane were present on at least two areas of the explant (Marks and Leopold, 1971).







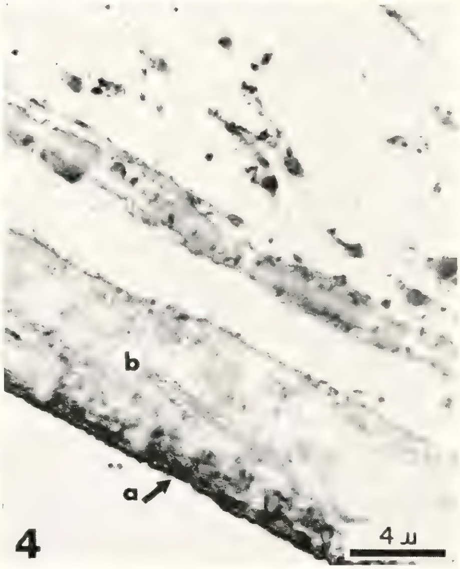


FIGURE 1. Leg regenerate with five successive cuticles resulting from five separate doses of ecdysterone. Secretion of most recent cuticle is still incomplete, and visualization is obscured by tissue.

FIGURE 2. Same specimen as Figure 1 after epidermis has retracted. Most recent cuticle is complete, and five cuticles are clearly visible.

FIGURE 3. Electron micrograph of inner of two cuticles secreted by a single leg regenerate. Surface of epidermal cell is visible at upper right. Epicuticle (a) is clearly distinguishable from less dense procuticle (b).

TABLE I

Comparison of responses obtained by administering a 1-μgd dose of ecdysterone in different ways

Number of exposures	Length of exposure (days)	Dose (μg/ml)	Total dosage (μgd)	Total days		Frequency of response (%)
				Exposed	Elapsed*	
1	1	1.00	1	1	1	16
2	1	0.50	1	2	5	25
4	1	0.25	1	4	13	20

* Includes time between repeated doses.

Two types of dosage were investigated, those in which the period of exposure was constant and the concentration of the hormone was varied and those in which the concentration was constant and the exposure was varied. Also, two types of exposure were used, continuous and interrupted. For the interrupted exposure, the hormone was injected for a given period and then the chambers were rinsed, refilled, and held for 3 days before the next exposure; this procedure was repeated until the total dose was given. The total dose received by an explant was expressed as the number of days of exposure multiplied by the concentration of the hormone in μg/ml of medium. Thus, the expression of dosage in microgram days (μgd) was common to all the experiments.

RESULTS

Since time-dose studies often involve long exposures (or as with interrupted doses, long periods during which the total dosage is delivered), it was necessary to establish that an effective dose of the hormone was maintained under the conditions of the experiment and that the explant retained its sensitivity to the hormone over the entire period of exposure. Therefore, three chambers, each containing two explants, were injected with ecdysterone to give a final concentration of 2.5 μg/ml and incubated for 30 days. At the end of this period, all the explants had well-developed cuticle. The medium was then removed from these chambers and injected into other chambers containing fresh explants. Cuticle appeared on all the new explants within 10 days. In another experiment, ecdysterone (2.5 μg/ml) was injected into a chamber containing two explants. The medium from this chamber was transferred after 7 days to another containing two more fresh explants and transferred to a third chamber after 7 more days. Then after 7 days in the third chamber, the medium was returned to the first chamber for 7 days (no new chambers were available) and finally on again to the second one. Within 10 days after the first dose, the explants in all three chambers showed evidence of cuticular deposition. Furthermore, when the explants were exposed to the medium the second time, they developed a second cuticle inside the first. Moreover, when a chamber containing fresh explants was injected with the aged

FIGURE 4. Electron micrograph of outer cuticle of same specimen as in Figure 3. Again, epicuticle (a) can be distinguished from procuticle (b). Similarity in the structure of the two cuticles is apparent.

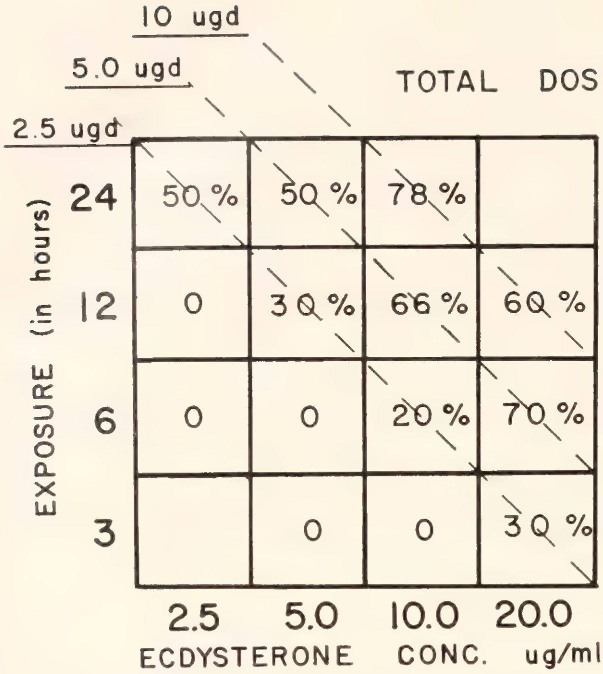


FIGURE 5. Diagram showing relative effect of concentration of hormone, time of exposure, and total dose (μgd) received on the frequency (%) of cuticle deposition by cockroach leg regenerates treated *in vitro* with ecdysterone.

medium some 35 days after the start of the experiment, cuticle appeared within 10 days. Superficially at least, this cuticle was no different from the first one in the series. The same experiment was repeated with a lower total dose (0.5 μg/ml for 5 days). After passage through three chambers, enough ecdysterone still remained in this medium to induce cuticular deposition on fresh explants in a fourth chamber.

Apparently neither metabolism by as many as 10 regenerates nor exposure of the hormone to breakdown over a period of 30 days was sufficient to reduce the dosages to a level below the threshhold. Marks and Leopold (1971) demonstrated that untreated regenerates used as solvent (H_2O) controls could be induced to respond to ecdysone treatment, even after 14 days *in vitro*. This ability to respond over a period of time was further explored by treating a series of chambers with ecdysterone (2.5 μg/ml). After five days, the treated medium was removed, and the chambers were rinsed and refed. As soon as cuticular deposits appeared on the explants, the chambers were refed and retreated with ecdysterone. This cycle was repeated until the tissues failed to respond. In this way, we were able to obtain as many as five separate cuticles from a single explant (Figs. 1 and 2), and the tissues apparently retained the ability to respond to treatment over 50 days.

One explant that had produced two cuticles was examined by electron microscopy: A section of the inner (second) cuticle (Fig. 3) was compared with a

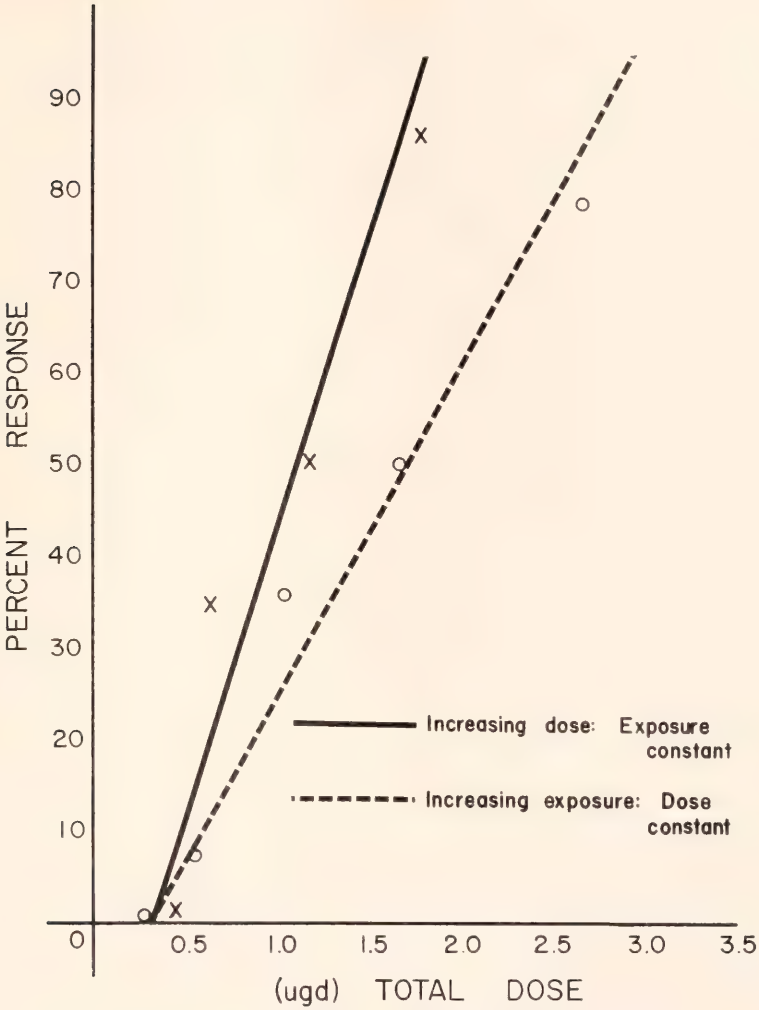


FIGURE 6. Graph showing the effect of the mode of delivery of a given total dose of ecdysterone on the frequency of cuticle deposition in cockroach leg regenerates.

section of the outer (first) cuticle (Fig. 4). Except for their position relative to the explant, it was difficult to distinguish between them. Each cuticle had an epicuticle (a) and a procuticle (b), evidence that they were the products of separate secretory cycles.

Once the long-term stability of the experimental system was established, a series of time-dosage tests was made. In the first series, high concentrations of ecdysterone (2.5 to 20 μg/ml) and short exposures (3 to 24 hr) were used. The minimum number of replicates for each determination was 10, and the results are given in Figure 5. The response to the hormone could be increased by increasing either the concentration or the length of exposure and was reasonably consistent in

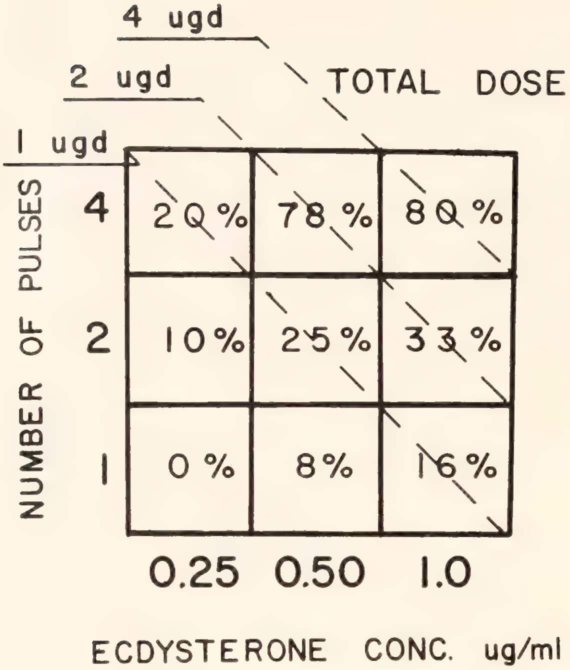


FIGURE 7. Diagram showing the relative effect of hormone concentration, number of exposures, and total dose received on the frequency (%) of cuticle deposition by cockroach leg regenerates treated *in vitro* with ecdysterone.

terms of total dose (μgd). When the exposure was 12 hr or less, the 50% response level lay between 2.5 and 5 μgd. At shorter intervals, the ecdysterone concentration required became so high, further experiments were impractical.

In the second series, the concentration of ecdysterone was lowered, and the period of exposure was increased. In one experiment, the period of exposure was held constant at 7 days, and the concentrations of ecdysterone varied from 0.05 to 0.50 μg/ml. In another experiment, the concentration was held constant at 0.50 μg/ml, and the periods of exposure varied from 12 hr to 7 days. The minimum number of replicates used to obtain each point was 10, and the results are given in Figure 6. The data show that time of exposure and concentration of hormone make roughly equal contributions to the effectiveness of the total dose. The dose required to give a 50% response lay between 1.0 μgd with a 7-day exposure and 1.4 μgd with a 3-day exposure. This was roughly half the dosage required for the short-term experiments.

Since the preliminary experiments showed that repeated large doses (10 μgd or greater) produced a series of separate cuticles, an additional series of experiments was made with repeated exposures of 1 day and concentrations of 0.25, 0.50, and 1.0 μg/ml. The minimum number of replicates was 12. After each dose, the chambers were washed with 3 changes of nutrient and held 3 days before the dose was repeated; as many as 4 such doses were given over a period of 13 days. The results are summarized in Figure 7. In no case did a recognizable cuticle appear until after the last dose was given. Apparently, the effects of the repeated doses are cumulative, and are independent of the dose given in a single exposure and of the time elapsed between the first and last exposure (Table I).

Discussion

Oberlander (1969a), working with wing discs of *Galleria*, reported that ecdysone remained active in cultures for as long as three days and found no evidence that the tissues metabolized appreciable quantities of the hormone. His findings and the results of the present experiments stand in sharp contrast to those of Shaaya (1969), Karlson and Bode (1969), and Ohtaki, Milkman, and Williams (1968), all of whom found that the half-life of ecdysone analogs in living insects was at best a few hours. The most obvious explanation for this difference between the *in vivo* and *in vitro* results is that the hormone is either rapidly inactivated or excreted by living insects; neither would occur to any great extent in an *in vitro* system. However, a recent study by Richman and Oberlander (1971) indicates that the situation may be more complex than this.

The fact that multiple doses of hormone will stimulate the production of multiple cuticles sheds some light on questions raised earlier by Marks and Leopold (1971) as to why the *in vitro* system produced cuticles containing only small amounts of procuticle. Since the cuticle produced by a second dose of hormone was essentially similar to the one laid down 12 to 15 days earlier, the mechanisms producing the cuticular materials must have retained the ability to operate *in vitro* over considerable periods. Moreover, since the explant was able to secrete as many as 5 similar cuticles, the incompleteness of the first one could not have occurred because of the inability of the explant to produce enough material. What occurred is apparently the normal response to ecdysterone stimulation, and the completion of cuticular deposition *in vivo* probably requires either an additional stimulus of a different type or another source of material. In any case, what occurred *in vitro* is only the first of a series of steps that leads to the formation of a complete cuticle, and this first step was repeated with each dose.

Oberlander (1969a, 1969b) found that a 2-hr exposure to ecdysone at a concentration of 3 μg/ml was sufficient to induce morphogenic changes in the wing discs of *Galleria*. In the present study, I found that the overall (7-day) threshold for cuticular deposition was 0.35 μgd, close to the 0.25 μgd required to induce morphogenesis in Oberlander's studies; with a 3-hr exposure, it was between 1.25 and 2.5 μgd. Thus, as the exposure decreased, the total dosage required to induce deposition increased, indicating that this system is somewhat less sensitive than Oberlander's. This lowering of the threshold with increasing exposures suggested that the accumulation of some substance or physiological event was taking place. Evidence that such a buildup did indeed occur was obtained from the experiments made with discontinuous doses. The subthreshold doses given over long periods eventually triggered a response; furthermore, when the total dose was the same, about the same response was obtained, whether the dosage was spread over 1, 5, or 13 days.

Since each chamber was washed three times and held for three days between exposures, it is difficult to believe that a quantitative buildup of the ecdysterone occurred unless it was tightly bound by the tissue. Thus, the relationship between concentration and exposure and the quantitative accumulation of interrupted doses suggested that it was the "event" rather than a substance that accumulated. Like the "events" resulting from exposure to ionizing radiation, these are apparently discrete and of an all or none nature.

Ohtaki, Milkman, and Williams (1968), working with *Sarcophaga*, assayed the concentration of ecdysone present at various stages of development and found that it never reached the level required to induce pupation in an isolated abdomen, but it was never entirely absent. They concluded that the presence of a low titer of hormone over a period of time resulted in the accumulation of a series of covert events that accumulated until they finally produced an overt response. The minimum period for the process of accumulation, regardless of the concentration of hormone, was 8.5 hr, which is in the same range as the 3 to 12 hr reported by Oberlander (1969a, 1969b) and the 6 and 12 hr required for a 50% response in my experiments. These results give strong support to the hypothesis of Ohtaki, Milkman, and Williams (1968). Such an all or none event might be either the tenacious occupation by the ecdysterone molecule of a receptor site on or within the cell or the passage into the cell of a single molecule of the "macromolecular factor" proposed by Williams and Kambysellis (1969). In the latter case, the ecdysterone would act to mediate the passage of the molecule through the cell membrane.

Since the entire series of events leading to cuticle deposition can be induced in the same explant as many as 5 times, it is apparent that once deposition has been induced and the cells have discharged their cuticular materials, they also discharge their accumulated events and are ready to start a new cycle, again responding in the same way as untreated tissue.

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Summary

Quantitative studies were made of the stimulation of cuticular deposition in cockroach leg regenerates *in vitro* by the molting hormone ecdysterone. Preliminary experiments showed that the hormone remained active and that the tissues remained sensitive *in vitro* for as long as 30 days. Large doses given one week apart resulted in the production of multiple cuticles, but small doses given at shorter intervals gave a single, cumulative response. Time-dosage studies showed that the concentration of the hormone and the length of exposure make roughly equal contributions to the effect of the dose.

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THE RESULT OF IMPROVED NUTRITION ON THE LANSING EFFECT IN *MOINA MACROCOPA*

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In a previous study (Murphy, 1970) it was noted that *Moina macrocopa* lived longer if the monoxenic culture medium contained a greater variety and larger amounts of water soluble vitamins. Similar effects of vitamins on the life span of other cladocera have been reported (Flückiger and Flück, 1950; Fritsch, 1953). Because *Moina macrocopa* is exceptionally short lived (Papanicolau, 1910; Wood and Smith, 1932) and therefore convenient for studies of aging, it was decided to investigate whether or not the Lansing Effect (Lansing, 1947) could be demonstrated with this species, and if so to find out if the extinction of old orthoclones could be prevented by improved nutrition.

The Lansing Effect refers to the finding that clones of a rotifer, *Philodina citrina*, raised through successive generations from old mothers, died out. The lines, which Lansing named orthoclones, were formed as follows. A fifth orthoclone, for example, would be started by isolating the young rotifer ($5F_1$) born of a mother on her fifth day of life. When this daughter rotifer is five days old, its daughter ($5F_2$), born that day, would be isolated. This process is repeated to produce $5F_3$, $5F_4$, *etc.* Thus, every animal in an orthoclone was born when its mother was the same age. In a fourth orthoclone all the mothers were four days old; in a tenth orthoclone, ten days old. Lansing showed that there were important differences between the young orthoclones and the old orthoclones. While the young orthoclone continued indefinitely in the culture conditions used, all orthoclones formed after the animals stopped growing eventually became extinct (Lansing, 1948). The older orthoclones displayed increasingly shorter life spans and an increasing tendency to die out.

Efforts to elicit the Lansing Effect in other organisms have met with failure. Comfort (1953) could not demonstrate any life shortening in 30 day orthoclones of *Drosophila subobscura*, and Fritsch (1956) could not demonstrate the effect with *Daphnia magna*, although he did not make an effort to test the oldest orthoclones.

Because each generation of an old orthoclone must be kept for virtually the entire life span of the animal before the next generation is born, studies of this type can, in practice, only be performed on animals with very short life spans, such as *Moina macrocopa*.

Our results show that there is a very striking Lansing Effect with *Moina macrocopa* and further that it can be modified by treating the older orthoclones with relatively high concentrations of inositol or liver infusion.

Methods and Materials

The monoxenic culture procedure used has previously been described in detail (Murphy, 1970). The medium is shown in Table I. Note that one component, pyridoxal, was omitted in the original description. All experiments were done at 20° C. Supplementary vitamins were made up at 10-fold concentrations, sterilized by filtration through a 20 micron porcelain filter cylinder and added directly to the petri dishes containing 9 ml of filtered medium. Dehydrated liver infusion (Oxoid) was made up 0.7 gram/100 ml, autoclaved and 1 ml, including some undissolved particles, was added per dish. The food organism was *Chlamydomonas reinhardii,* Indiana U. strain #90 grown in the medium of Levine and Ebersold (1958) enriched with one gram per liter of N-Z-Case peptone (Sheffield Chemical Co.) and with agar omitted. The animals were transferred three times per week.

Fritsch (1956) when using *D. magna,* which produced a clutch of eggs every two days, defined an orthoclone on the basis of clutch number. This is convenient and possibly more meaningful than chronological age when referring to cladocera (Ingle, Wood and Banta, 1937). We have followed the same terminology.

Table I

Composition of the medium (mg/liter). For directions on how to make up this medium, see Murphy (1970)

Calcium acetate · X H_2O	59.0
Potassium penicillin (U.S.P.)	215.0
Streptomycin sulfate (U.S.P.)	7.0
Bovine albumin, fraction V (Armour)	67.0
Calcium pantothenate	7.0
B_{12}	0.0003
Thiamin	0.6
Riboflavin	0.4
Nicotinamide	1.3
Folic acid	3.3
Biotin	0.3
Putrescine	0.3
Choline	5.0
Inositol	11.0
Pyridoxal	5.0
Hutner's Trace Elements	0.3 ml.

Moina macrocopa, under the basic conditions used in this study, is a highly variable animal. It usually lives about ten days to two weeks and produces about 100 parthenogenetic young divided into five clutches. Even though some animals may die at any time as if a random death process were operating, under improved nutritional conditions, the number of deaths in all age groups is diminished. As there is no apparent way to distinguish between the causes of death in younger and older animals, all deaths are included in the data. This makes larger experimental samples necessary to reach statistically significant levels, but the overall conclusions are not affected.

Animals chosen for transfer have been selected on the basis of the largest clutches from the most actively moving adults. This would be expected to

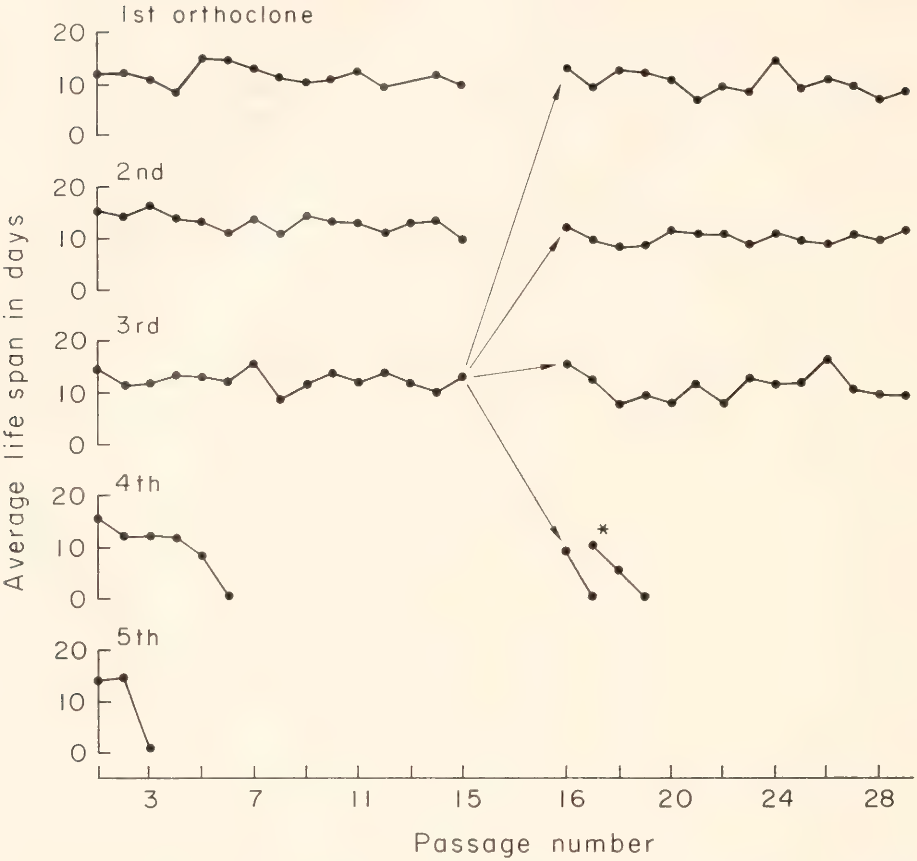

FIGURE 1. The average life span (five animals/datum) of successive passages of the first through fifth orthoclones of *Moina macrocopa.* The fourth and fifth orthoclones die out thus demonstrating the Lansing Effect. At passage 15 of the third orthoclone, the experiment was restarted using the progeny of one animal. The animals indicated in the datum marked * were the young of a mother raised with extra inositol.

eventually yield a clone superior to the original but no such effect was found during the limited time span of this study. On the contrary, it is necessary to select the strongest animals to maintain any clone in continuous passage.

RESULTS

Demonstration of the Lansing effect in Moina macrocopa

Figure 1 shows the average life spans in days of orthoclones from the first through fifth clutches. Six clutches are rare under our conditions. The data are shown as averages of five animals, but it should be noted that nearly every datum contains animals that died after 4 to 5 days or animals that lived 16 to 18 days. In spite of the variation it is clear that the first, second and third

TABLE II

The effect of inositol on the average life span and average number of young per lifetime on fourth orthoclone Moina macrocopa. *(Each datum represents the mean of five animals)*

	Inositol concentration (mg/liter)	Passage 1		Passage 2		Passage 3	
		Life span	Young	Life span	Young	Life span	Young
Control	11	5.6	4.8	died out			
Inositol pulsed*	61 or 11	7.9	46.5	13.6	91.6	9.5	85.0
Vitamin mixture pulsed*†	61 or 11	10.0	49.8	11.0	50.8	—	—
Inositol continuous	61	9.9	42.2	15.2	69.6	9.4	51.4

* Pulsed indicates that the animals were in the stronger concentration for 24 hours twice a week.

† This group had extra riboflavin, folic acid, calcium pantothenate and nicotinamide along with inositol.

orthoclones were definitely established at the end of the experiment while the fourth and fifth died out.

The slight apparent decline in life span shown by the first and second orthoclones is an artifact and did not continue. The third orthoclone has been continued for over 28 generations with no diminution of life span, average number of young produced, or apparent vigor.

The effect of inositol and some water soluble vitamins on old orthoclones

In a previous study (Murphy, 1970) it was noted that shortening of life span due to avitaminosis did occur in the daphnidae possibly because the biosynthetic mechanism leading toward egg production is capable of drawing so heavily on its reserves that the animal is thrown into pathological deficiency. We had also noted that species that could barely survive under the conditions being used were the best indicators of an improvement in the medium because they quickly showed such sharp endpoints as living or dying, or bearing or not bearing young.

On the basis of these previous experiments it was decided to test fourth orthoclone animals with higher concentrations of some of the components of the medium. In a preliminary experiment, a mixture of five was chosen and used at a concentration of five times that in the regular medium.

The first substances tried were inositol, riboflavin, folic acid, calcium pantothenate and nicotinamide. The initial studies showed that the mixture was toxic after 24 hours. For this reason animals were treated with 24 hour pulses twice a week. On this regime, the fourth orthoclone became established. A preliminary experiment indicated that inositol alone was responsible for the result. Table II lists the data for life span and number of young for three orthoclone passages under several nutritional regimes. There is no significant difference among the groups with added inositol except that the number of young produced after two 24 hour treatments each week shows a consistently higher average. Although the difference is significant only at the $P = < 0.2$ level, this routine was chosen because we felt the animals also looked more vigorous.

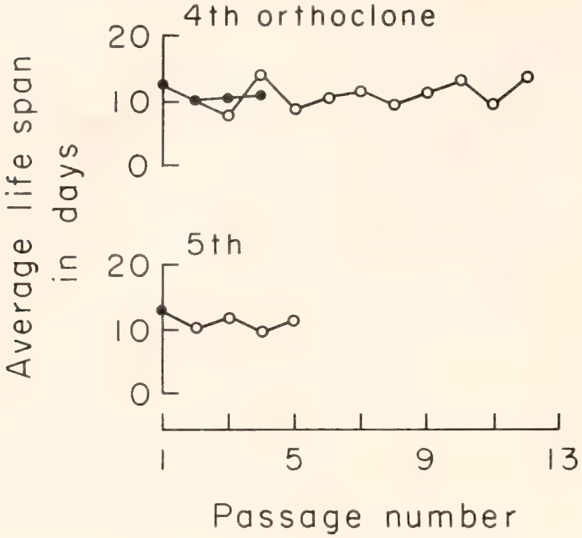

FIGURE 2. The average life span (five animals/datum) of successive passages of the fourth and fifth orthoclones of *Moina macrocopa* after extra inositol was added to the medium for 24 hours, twice a week. This figure should be compared with the fourth and fifth orthoclones in Figure 1. ●———● indicates that the medium also contained extra amounts of vitamins; ○———○ indicates no extra vitamins.

The pulses of inositol at 61 mg/liter for 24 hours, twice a week, alternated with a concentration of 11 mg/liter the rest of the time. As can be seen from Figure 2, the fourth and fifth orthoclone could now be established.

Test of seven more vitamins on the fifth orthoclone

Using the same arguments as those used in previous experiments, pulses of the remaining six water soluble vitamins (Table I) at five times the concentrations used in the regular medium and a water soluble form of vitamin E (DL-α-tocopherol phosphoric acid ester, disodium salt) 1 mg/liter were tried singly and in combination on fifth orthoclone animals already being supported by biweekly pulses of inositol. The results showed no consistent increase in life span over inositol alone. The fifth orthoclone's existence was extremely tenuous since generally only one of the five animals lived long enough to produce a fifth clutch of young.

Effect of liver infusion

Provasoli, Conklin and D'Agostino (1970) found liver infusion (Oxoid) to have an enhancing effect on *Daphnia magna* and *Moina macrocopa*. The concentration suggested by Douglas Conklin, Haskins Laboratories (personal communication) of 700 mg/liter added to the regular medium produced a most remarkable and almost immediate improvement in the fifth orthoclone cultures (Table III). The animals became more active and more deeply pigmented. The number of young born in a lifetime sharply increased and the females had their clutches of young a

TABLE III

The effect of liver infusion (Oxoid) 700 mg/liter on the fifth orthoclone of Moina macrocopa. *(Each datum represents the mean of five animals)*

	Passage 1		Passage 2	
	Life span	Total young*	Life span*	Total young*
Inositol pulsed 11–61 mg/liter	11.4	82.2	10.8	94.3
Liver infusion continuous	12.0	126.7	12.5	136.2

* The values for liver infusion are all significantly greater than those for inositol at the $P < 0.05$ level except for the lifespan values in passage 1. The average young/hatch in liver infusion is 24.9.

day earlier than the pulsed inositol controls. Although the life span did not markedly increase, the fifth orthoclone appears to have become solidly established because the young were born earlier.

Our preliminary results with a sixth orthoclone in liver infusion give little hope for its survival.

DISCUSSION

The interpretation that the Lansing Effect in *Moina macrocopa* is brought about by relative nutritional deficiency seems quite clear. It is tempting to compare the conditions which Lansing (1947) used to culture rotifers and speculate as to whether he too was observing a nutritional effect.

Lansing used a single species of alga, *Chlorella vulgaris*, as a food organism, culturing the rotifers in a mineral medium without added nutrients and transferring them daily. Probably some bacteria were present, but it is doubtful if they could have reproduced well enough in this environment to become a significant supplemental food source. Experiments with *Daphnia pulex* indicate that *Chlorella pyrenoidosa* or *Chlamydomonas reinhardii* alone are not adequate as food organisms (Taub and Dollar, 1968). Single species of algae may also be inadequate for rotifers. This suggests the possibility that Lansing's experiments may have been carried out under suboptimal nutritional conditions.

Whether the Lansing Effect has the same mechanism in rotifers as it does in a crustacean does not alter the fact that there exists in both organisms a nongenetic, transmissible, cumulative premature aging phenomenon operating over many life spans.

Other examples of non-genetic parental influence on life span have been recorded. Papanicolau (1910) observed a gradual reduction in life span of first orthoclones of *Moina rectirostris* and *Simocephalus vetulus* in the laboratory. Rockstein (1957) has shown that old houseflies produce young with short life spans, a result indicating that a Lansing Effect might have been operating. His animals were raised initially on sugar, milk and yeast and switched to sugar alone when adults. Recently, Zamenhof, van Marthens and Grauel (1971) have observed in rats that protein restriction one month before mating and continued through pregnancy reduces total brain DNA in the young. This defect is transmitted to the F_2 generation even though nutritionally adequate diets were reinstituted at the time of birth of the F_1. The term "cryptic malnutrition" is used to describe the phenomenon.

The question of whether a further major increase in life span might be possible with *Moina macrocopa* remains open. The most productive and long-lived animals reported by Wood and Smith (1932) are very similar to our best in liver infusion. Stuart and Banta (1931) and Stuart, McPherson and Cooper (1931) report an average clutch size of 17 or less, lower than that found in our study (24.9 young/clutch). Terao and Tanaka report shorter life spans (1930) and lower reproduction rates (1928) at 20° C. However, the preliminary finding that the sixth orthoclone will not survive should probably be considered evidence that our animals are still not under ideal nutritional conditions. At any rate, the Lansing Effect has not been abolished. It is possible that the lack of a detectable Lansing Effect in Comfort's (1953) experiments with *Drosophila subobscura* was the result of the ideal nutrition of his cultures.

Finally, the finding that the Lansing Effect is not confined to rotifers raises the possibility that it may underlie the not uncommon experience of having cultures of various kinds fail after numerous apparently successful passages.

SUMMARY

Lansing showed that clones of rotifers raised through successive generations from old mothers will die out (Lansing Effect). We have found that the phenomenon also can be shown in a crustacean, *Moina macrocopa*, cultured monoxenically on *Chlamydomonas reinhardii*. Furthermore, we have found that the Lansing Effect can be modified and partly prevented by exposing the animals to relatively large amounts of inositol or liver infusion. It is suggested that the Lansing Effect in *Moina macrocopa* is the result of a long range, transmissible, cumulative, premature aging phenomenon due to relative nutritional deficiency.

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JUVENILE HORMONE IN RELATION TO THE LARVAL-PUPAL TRANSFORMATION OF THE CECROPIA SILKWORM

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Juvenile hormone (JH) has long been known to oppose or prevent the metamorphosis of insects (Wigglesworth, 1934). In the Lepidoptera its most familiar effects are on the pupal-adult transformation where one observes the formation of a second pupa or pupal-adult intermediates (Piepho, 1952; Williams, 1961). Its effects on the larval-pupal transformation of Lepidoptera were first demonstrated by Piepho (1939, 1942) by the implantation of active corpora allata into final instar larvae of the wax moth *Galleria mellonella.* At the subsequent molt either a supernumerary larva or a larval-pupal intermediate was produced. In experiments on *Galleria* Sehnal (1968) confirmed the effects of implanted corpora allata and duplicated them by the application of Cecropia-JH to final instar larvae (Sehnal and Meyer, 1968).

It has been difficult or impossible to cause these effects on other species of Lepidoptera (Bounhiol, 1938; Piepho, Böden, and Holz, 1960; Williams, 1961). Fukuda (1944) was able to produce supernumerary *Bombyx mori* larvae but only by implanting both active corpora allata and active prothoracic glands into final stage larvae. Williams (1961) could not affect the metamorphosis of last instar *Hyalophora cecropia* larvae by the implantation of active corpora allata. Staal (1967) found that implantation of active glands into a larval tubercle caused the retention of only that tubercle in an otherwise normal pupa. Finally, Willis (1969) has reported the retention of larval tubercles on certain Cecropia pupae thought to be infected with *Nosema,* a microsporidian from which a material with JH activity has been extracted (Finlayson and Walters, 1957; Fisher and Sanborn, 1962, 1964).

Several years ago, when potent JH analogues became available, it seemed likely that application of high doses at the proper time might be able to block the larval-pupal transformation of the Cecropia silkworm. My preliminary studies (Riddiford, 1968b) showed that high daily doses of these materials during the fifth larval instar failed to provoke an extra larval instar although in many individuals certain larval characters were preserved. Even more surprising was the finding that JH application to mature larvae which had completed spinning prevented diapause and interfered with the larval-pupal transformation of the internal organs without affecting the metamorphosis of the integument.

The present paper reports the details of a study of the effects of exogenous JH on the larval-pupal metamorphosis of the Cecropia silkworm.

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MATERIALS AND METHODS

Experimental animals

Cecropia were reared on wild cherry trees (Telfer, 1967) and in the laboratory either on an artificial diet (Riddiford, 1968a) or on cherry leaves sprayed with an antibiotic mixture (Riddiford, 1967). For the duration of the fifth instar and the prepupal period, the animals were reared at 25°–26° C under 17 hours of daily illumination.

Juvenile hormone applications

A mixture of synthetic juvenile hormone analogues was prepared by bubbling hydrogen chloride gas through farnesenic acid dissolved in ethanol (Vinson and Williams, 1967); this mixture (JH-A) was used in most experiments. When injected into Polyphemus pupae (Williams, 1961), 10 μg gave a + 3 pupal-adult intermediate; 20 μg of a second preparation of the same mixture was necessary for a + 3 score. Therefore, in the presentation of the data a dose of the second preparation was equated to half that of the first preparation.

In certain experiments, I also made use of a synthetic Cecropia C18-JH (JH-C) synthesized by Corey, Katzenellenbogen, Gilman, Roman, and Erickson (1968). In the Polyphemus pupal assay 0.05 μg of this compound gave a + 3 score.

The hormonal materials in 1 to 5 μl of acetone ("Nanograde," Mallinckrodt) were applied topically along the dorsal midline of the meso- and metathorax and, less often, to the abdomen of fifth instar larvae or prepupae. Daily applications were always made at the same time of day, usually in the late morning, and were continued until the larva stopped feeding and emptied its gut.

Surgical procedures

The techniques of brain removal and implantation were as previously described (Williams, 1946, 1952; Schneiderman, 1967). After surgery, the pupae were placed at 25° C and observed at biweekly intervals to detect the initiation of adult development.

RESULTS

(1.) *Topical application of juvenile hormone at successive stages in the fifth larval instar*

(a) *External morphology of pupae obtained from treated larvae.* Fifth instar Cecropia larvae exposed to JH formed pupae which often retained certain external larval characters. The scoring system outlined in Table I was based on 67 individuals which had been treated with specific doses of JH-A and JH-C during the fifth instar. The dorsal tubercles, particularly in the region where the hormone was administered, proved to be most sensitive to topical application (Fig. 1A). With increasing dosage, the spiracles and patches of dorsal epidermis were next affected, followed by the thoracic legs, the labrum, and the larval prolegs. Finally, the highest dose of 20 μg JH-A daily (higher doses were lethal) produced a larval-

TABLE I

Scoring system for evaluating external larval characteristics reformed on Cecropia pupae after juvenile hormone treatment of larvae

0	Normal pupa
+1	Traces of dorsal and supraspiracular tubercles retained near site of application on meso- and/or metathoracic tergites and often extending posteriorly to first or second abdominal segments.
+2	Partial to complete reformation of dorsal and supraspiracular tubercles on thorax and anterior abdominal segments; occasional patches of larval cuticle on tergites; many spiracles of larval type.
+3	Complete reformation of dorsal and supraspiracular and sometimes of infraspiracular tubercles on thorax and on at least the 3 anterior abdominal segments; extensive patches of larval cuticle on meso- and metathoracic tergites; larval-type spiracles; larval claws retained on thoracic legs.
+4	All or nearly all tubercles retained; patches of larval cuticle on tergum and at sites of larval prolegs; spiracles and labrum of larval type; tips on pupal antennae and on thoracic legs larval.
+5	Head and thorax with larval characters throughout; abdominal tubercles and prolegs retained. Remainder of abdomen with pupal cuticle.

pupal intermediate which retained a larval head and thorax and larval tubercles on a pupal abdomen.

Most of the treated larvae formed diapausing pupae which, after 3 or more months of chilling at 5° C, formed essentially normal adults when placed at room temperature. It was of considerable interest that the sites which retained larval cuticle in the pupa were covered with pupal cuticle in the moth (Fig. 1B).

(b) *Daily applications.* As seen in Table II, daily applications of either JH-A or JH-C from the beginning of the fifth instar caused the formation of pupae which showed the retention of certain larval characters. The higher the dose, the more affected were the pupae. Of the 21 treated animals, 10 did not live to adult

TABLE II

Effects on pupae of daily applications of juvenile hormone materials to Cecropia larvae throughout the fifth instar

Daily dosage (in 1 μl acetone)	Number treated	Days before spinning	Number pupae	JH effects*
0 [Controls]	3	13–15	3	0
		Juvenile Hormone Analogue Mixture		
1 μg	3	14–17	3	0, 0, +0.5
5 μg	3	16–20	3	+2, +2, +3
10 μg	7	13–16	7	+2(2), +3(5)
20 μg	2	15–17	2	+4, +5
		Cecropia C-18 Juvenile Hormone		
0.5 μg	2	17	1	+2.5
1 μg	4	12–21	3	+1, +1, +4

* System for scoring of external characters is presented in Table I.

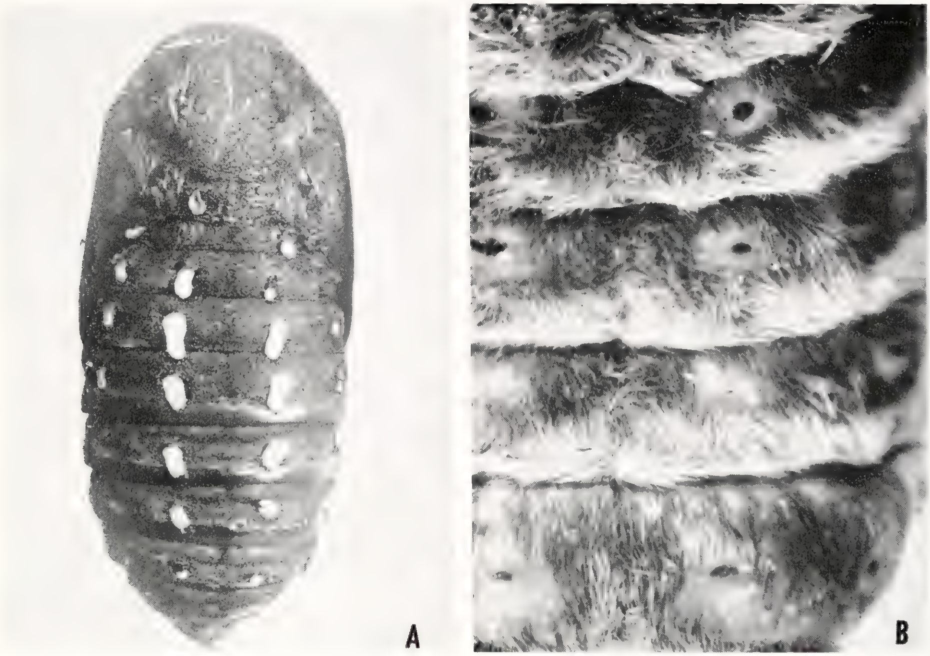

FIGURE 1. (A) Cecropia pupa showing retention of larval tubercles on metathoracic and abdominal tergites after application of 10 μg of a mixture of juvenile hormone analogues daily during the fifth instar; (B) A portion of the abdominal tergites of the adult moth which developed from the pupa in Figure 1A. Note the spots of pupal cuticle at the sites where larval tubercles had been on the pupa.

emergence. Three other pupae (two receiving 5 μg JH-A and one 0.5 μg JH-C) did not enter diapause and emerged as adults 30 to 50 days after pupation.

JH-A prevented the complete evacuation of the gut (the normal prelude to spinning) in at least 5 of the 15 treated individuals. All these larvae subsequently spun cocoons of which five were very thin with abnormally large valves. Though the cessation of feeding and a partial evacuation of the gut occurred at the normal time in larvae treated with JH-C, the onset of spinning was delayed by 8 to 10 days except for one individual that had received 1 μg. Furthermore, the time between spinning and pupal ecdysis was usually 1.5 to 2 times the normal 10 days. In an extreme case, a larva which received daily applications of 1 μg JH-C failed to empty its gut or to spin. It lived 42 days after the cessation of feeding, but showed no signs of pupal differentiation. These findings strongly argue that gut evacuation and the initiation of spinning can be delayed or even blocked by JH.

Retention of larval integumentary characters was also seen in eight pupae formed from larvae that received daily treatment with 10 or 20 μg JH-A beginning on day 5 or 10 of the fifth instar and continuing until spinning. Application of 20 μg JH-A for three consecutive days prior to spinning was sufficient to cause a +2 to +3 reaction in the resulting pupa.

Although JH-C produced the same type of results as did JH-A (Table II), much higher doses were necessary in terms of the activities of the two materials in the Polyphemus pupal assay. This difference is presumably due to a more rapid inactivation of JH-C by mature larvae.

(c) *Single applications.* A single dose of JH-A was administered to a series of 41 individuals. These larvae at the time of treatment were at successively later stages in the fifth instar, ranging from pharate fifth stage (−1 day) to the mature fifth stage at the time of gut evacuation (11.5 to 14th day). The results summarized in Table III show that the dorsal tubercles were sensitive to the hormone up until the emptying of the gut. The time of maximum sensitivity seemed to be about 2 to 4 days prior to gut evacuation. Larvae treated with 150 μg JH-A on day 8 and 9 emptied their gut on day 11 to 12 and began spinning one to two days later. Applications before this time were much less effective, presumably because of the inactivation of the hormone.

Table III

Effects on pupae of a single dose of a mixture of juvenile hormone analogues applied to fifth instar Cecropia larvae

Day of 5th instar	15 μg		60 μg		150 μg		250 μg	
	Number treated	JH effects*	Number treated	JH effects*	Number treated	JH effects*	Number treated	JH effects*
−1							2	0,0
0							2	+1,**
1							2	**
5	1	0			2	0, +1		
6	2	0, +1			2	+1, +1		
7	2	0, 0	2	0, 0	2	+2, +2		
8	2	0, 0	1	+2	2	+4, +4		
9	1	0			1	+4	1	+4
10	2	0, 0	1	+2	1	+3		
11					1	+4		
12§	1	+1	1	+3	2	+2, +3		
13§			1	+2				
14			2	+1, 0	2	0, +1		

*System for scoring external larval characters reformed on the pupa is presented in Table I.

** Death before pupation, usually at time of gut evacuation.

§ Although normal time for gut evacuation at 26° C is day 11.5 to 14, these individuals had not emptied their guts at the time of application.

When JH-A was applied on days 8, 9, or 10 of the fifth instar, occasional individuals spun thin cocoons or flat pads of silk. These anomalies were usually associated with a 1 to 3 day delay between gut evacuation and the onset of spinning. I found that the time of evacuation can be predicted from the weight of the larva. Thus, during the fifth instar there is a steady increase, then a plateau. Two days after the beginning of the plateau, evacuation occurs. When 150 μg JH-A was applied one day after the beginning of the plateau, evacuation was delayed for 1 to 4 days. When the hormone was applied to larvae in the process of gut evacuation, there was no delay in the initiation of spinning and normal cocoons were spun.

(2.) *Topical application of juvenile hormone to spinning larvae*

Once a larva has emptied its gut and initiated spinning, the larval integument becomes very resistant to JH. Thus, after the application of 250 or 500 μg JH-A on the first day of spinning, only an occasional individual formed a pupa which retained one or two dorsal tubercles near the site of application. After the first day even these structures could no longer be affected. All the pupae given JH as spinning larvae entered diapause and after several months of preliminary chilling developed into normal moths when placed at room temperature.

TABLE IV

Effects on the incidence of diapause and on adult differentiation of a single application of 150 μg of a mixture of juvenile hormone analogues to Cecropia larvae and prepupae between spinning and pupal ecdysis

Time of application (days after gut evacuation)	Number treated	Number failing to diapause	Juvenile characters in resulting moths†						
			External*	Internal**					
				Thorax	Fat body	Gut	Gonads	Accessory glands	Abdominal intersegmental muscles
0	3	1	0	2§	5	3	4	1	2
1	3	0	0	0	0	0	0	0	0
2	7	4	0	2	3.8	2.6	3	2.2	2.3§
3	3	2	0	2.8	4	2	3.5	1	1.5
4	2	2	0.5	4	4	3§	4.5	2.8	5
Ocellar retraction									
5	2	2	0.2	3§	4	3.5§	4	3	4§
6	2	2	1.3	3.5	5	4.5	4.5	3.8	5
8	1	1	0.5	2.5	3	1	3	3	1
White prepupa									
9	2	2	0	2	4	1	3	1	2

† When there is more than one treated individual, an average score is used. This average is based only on the scores for nondiapausing individuals.

* Scoring of external characteristics based on pupal assay for juvenile hormone, Williams (1961).

** Scoring of internal characteristics based on Figure 2.

§ At least one individual showed retention of larval characters.

(3.) *Topical application of JH to post-spinning larvae and to prepupae*

A single application of JH at any time from the cessation of spinning until pupal ecdysis had no effect on the integument of the resulting pupa. A most surprising finding was that many of these pupae failed to diapause so that moths were formed 24 to 30 days after pupal ecdysis. Furthermore, dissections of 2-day-old pupae which had been treated with 150 μg JH-A early in the prepupal period revealed that many internal organs retained larval characteristics. For example, the hindgut retained the larval diverticula, the gonads were much smaller than in normal pupae, the larval thoracic and abdominal muscles were mostly intact, and there were 7 instead of the usual 6 abdominal ganglia. In normal pupae at the time of pupal ecdysis, the morphology of the viscera is pupal except for a small amount of degenerating larval musculature and the subsequent fusion of abdominal ganglia to reduce the number from 6 to 5. Consequently, it is clear that after JH treatment of the early prepupa, the metamorphic processes of the viscera had been severely retarded with little or no effects on the pupation of the integument.

(a) *Prevention of diapause.* A single dose of 1, 5, 10, 25, 50, 100, 150, or 300 μg JH-A was applied to each of 91 individuals at various times ranging from gut evacuation until pupal ecdysis. Table IV summarizes the effects of 150 μg JH-A as a function of the time of application.

In the first three columns of Table IV we see that during the period of gut evacuation and spinning, the larvae were very insensitive to JH-A. Most of the resulting pupae diapaused and, after several months of preliminary chilling, developed into normal adults when returned to 25° C. However, when the application was delayed until after spinning, the resulting pupae failed to diapause. This same result was observed when 50 μg JH-A was applied on day 3 or subsequently up to 12 hours after pupal ecdysis.

TABLE V

Endocrine activity of brains of freshly pupated individuals after juvenile hormone treatment during the prepupal period (Tested by implantation into brainless diapausing Cecropia pupae)

Time of brain removal after pupal ecdysis	Number implants	% Donor pupae developing	% Host pupae developing	Days for initiation of development of host pupae
Juvenile hormone-treated prepupae				
4–12 hours	15	40	47	19, 33, 38, 111, 141, 150, 151
14–18 hours	5	60	20	160
24–26 hours	9	67	22	9, 190
30–36 hours	4	75	25	9
Untreated prepupae				
4–12 hours	3*	0	0	—

* For a more extensive control series, see Table III in Williams (1952).

The obligatory diapause of the Cecropia pupa is due to the cessation of secretion of the brain's prothoracicotropic hormone prior to pupal ecdysis (Williams, 1952). Therefore, it was of importance to determine if the prevention of diapause by JH was due to an interference with this programmed shutdown of the brain or to a prothoracicotropic effect of the applied JH (Gilbert and Schneiderman, 1959; Williams, 1959). To test their endocrine competence, brains from fresh pupae which had been treated with JH-A at various times during the prepupal period were implanted into brainless diapausing Cecropia pupae. As seen in Table V, brains removed within 12 hours after the pupal ecdysis stimulated development of about 50% of the brainless hosts. As the time between ecdysis and brain removal increased, the number of developing hosts declined, thereby signaling a decline in the hormonal activity of the brain. This decrease was matched by an increasing percentage of development among the brainless donors, indicative of release of the prothoracicotropic hormone prior to the brain's excision.

The non-diapausing pupae formed pharate moths which were apparently normal to external examination, the only self-evident abnormality being their failure to undergo ecdysis. Upon dissection under Ringer's solution, all these individuals revealed the preservation of many internal pupal characters.

(b) *Internal morphology of adults obtained from exposure of prepupae to JH.* Figure 2 diagrams the scoring system for the internal organs of the "adults" which developed from the treated prepupae. Zero signifies full adult development, whereas organs scored as +5 remained completely pupal. A total of 62 individuals were scored in this manner.

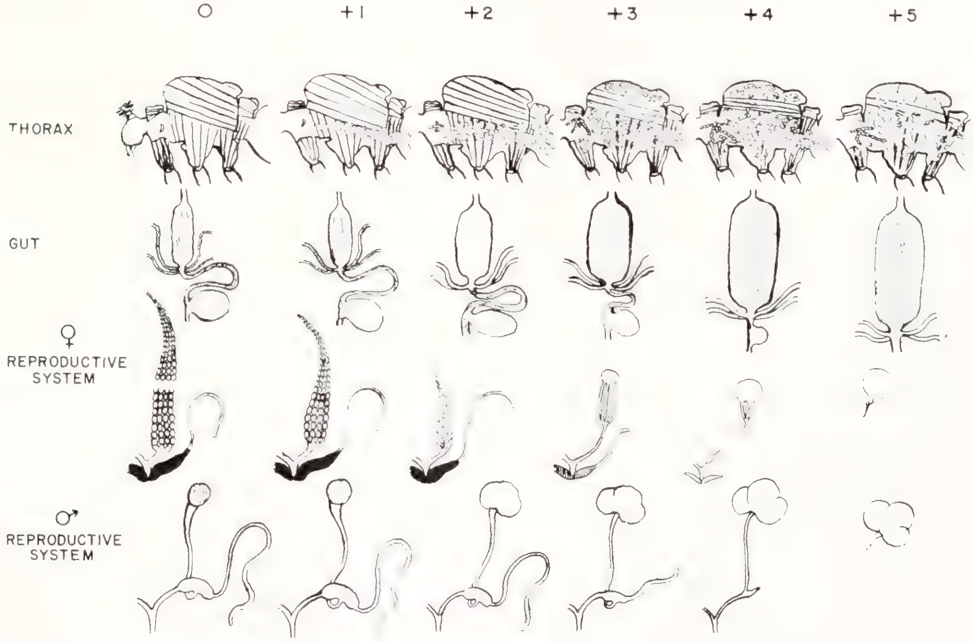

FIGURE 2. A diagrammatic representation of a scoring system for the juvenile hormone effects on the metamorphosis of the internal organs; 0 signifies the adult form; +5 designates the pupal form.

Of the three regions of the body, the organs within the head were least affected. During adult development, the brain normally undergoes great enlargement of the optic lobe area due to the axonal ingrowth from the developing compound eyes. Since the eyes always underwent full development in treated animals, the brain showed the same gross appearance as in normal adults.

Most of the moths formed from the JH-treated prepupae failed to emerge. Upon dissection they showed faulty development of the flight muscles. The tergosternal muscles were most affected, followed by the ventral longitudinal and, finally, the dorsal longitudinal muscles. Except in +5 individuals the leg musculature was usually present but reduced in volume. Concomitant with a decrease in the thoracic muscles was an increased quantity of fat body in the thoracic cavity. Juvenile hormone treatment also caused the retention of the prothoracic glands. In +3 individuals these glands were approximately half the size of the pupal organs.

In normal Cecropia moths the status of the abdominal organs is as follows (see left hand column in Fig. 2): The midgut is short and thin-walled, and communicates with a long coiled hindgut which culminates in a large rectal pouch and associated caecum (Judy and Gilbert, 1969). The Malpighian tubules empty into the gut at the juncture of the mid- and hindgut. In the moth these tubules are thin, straight-sided, coiled, and full of chalky tan meconium. The adult fat body is arranged in long, unbranched strips, each of which has a small trachea as a mid-rib. In normal female moths, most of the abdomen is occupied by the ovaries. The ovarioles contain about 250–300 mature chorionated eggs as well as numerous other oocytes in various stages of maturation (Telfer and Rutberg, 1960). The female accessory glands are well-developed and contain a dark reddish-brown glue. In normal male moths, the testes are small, bright yellow, and contain a small number of mature sperm. The vas deferens leads from each testis to the paired seminal vesicles which contain most of the sperm and receive the contents of the long tubular accessory glands. Ducts from the seminal vesicles come together to form the short median ejaculatory duct.

The abdomen of normal moths contains four ganglia. These innervate the three wide bands of the intersegmental muscles which line each side of segments 4 through 6. Within 48 hours of adult emergence, these muscles ordinarily are eliminated (Finlayson, 1956; Lockshin and Williams, 1965).

As is evident from Figure 2, JH application to prepupae had pronounced effects on the adult differentiation of all abdominal organs. Effects on the gut ranged from the full retention of pupal characteristics, with a large midgut and short straight hindgut, to the normal adult condition. Also, the Malpighian tubules varied from the knobby pupal type which were filled with meconium to tubules of the adult type.

In the most affected males (+5), the testes were completely pupal. Each gonad was divided into four thin-walled compartments which were filled with germinal cysts showing no elongation or spermatogenesis. A +3 male testis was intermediate in size and contained both round immature cysts and elongate cysts with maturing spermatocytes but only occasional bundles of mature sperm. The +3 male accessory glands were small and contained little or no secretions. Similarly, the most affected females (+5) retained pupal ovaries. More common was the +3 condition in which the ovarioles were well-formed but contained a few immature oocytes and no mature chorionated eggs. The +3 glue glands were small and filled with a yellowish fluid. In both sexes the development of the gonads was usually more retarded than that of the accessory glands.

Effects on the fat body were scored as +5 when this tissue was arranged in flattened branching ribbons which lacked a central trachea. The score +3 indicates that approximately half the cells were dissociated as is typical of early adult development. In animals scored +3 to +5, five separate abdominal ganglia were found, signaling a failure of the normal fusion. The nerve cords of these same individuals also lacked the heavy tracheation normally seen in adults. The intersegmental muscles were retained in Cecropia scored as +3 or higher.

Occasionally, as noted in Table IV, certain internal organs retained larval characteristics. Among these were the following: part of the musculature of the midgut walls, the diverticula of the larval hindgut, persistence of large portions of the silk glands and of the intersegmental muscles in the anterior abdomen and occasionally in the thorax, and retention of labial glands of the larval type.

(c) *Prevention of internal metamorphosis.* Those treated Cecropia which underwent pupal diapause invariably developed into normal adults after several months of preliminary chilling. By contrast, when JH treatment caused immediate development without pupal diapause, the resulting moths ordinarily showed the juvenile internal characteristics listed in the last 6 columns of Table IV. The metamorphosis of the viscera was severely affected by hormone application on day 4 or 5 after gut evacuation. In many of the resulting moths, larval viscera were often preserved. Late in the prepupal period the internal organs were less affected, and no larval structures remained in the adults. The same experiments testing the lower dose of 50 μg JH-A on 31 individuals showed essentially similar results. The effects were nearly as pronounced as after 150 μg except on day 6 and 7 when the molting fluid presented a barrier to the penetration of topically applied hormone (Williams and Sláma, 1966).

Ocellar retraction signals the onset of the prepupal period, whereas the final phase of this period is accompanied by loss of green integumentary pigment ("white prepupa"). The effects of graded doses of JH-A were examined at these two particular stages which are designated as "early" and "late" prepupae. The results summarized in Table VI reveal that most of the viscera were generally more affected after early application. Also, the prepupae treated early showed a more consistent dose-response relationship than did those treated late. Yet the late application of JH-A was more effective in the prevention of diapause. When JH was applied to white prepupae, larval structures were not retained since by this stage most larval tissues were in a stage of advanced histolysis or had nearly completed their pupal transformation.

As noted above, normal pupal-adult transformation of the viscera occurred in those individuals which diapaused after JH treatment. Similar results were obtained with five Cecropia treated with 50 μg JH-A as early prepupae and forced to diapause by removal of their brains immediately after pupal ecdysis. All developed into normal moths after an injection of 20 μg α-ecdysone 3 months later. Thus, the effect of JH on the internal organs decayed in time, suggesting that the only action of the hormone might be on the brain to avert the normal onset of pupal diapause.

This hypothesis was tested on a series of 10 normal Cecropia pupae by the injection of 20 μg α-ecdysone on 4 successive days beginning 12 hours after pupal ecdysis. The pupae promptly initiated development and emerged about 26 to 30 days later. All were normal adults both externally and internally. Thus, in the JH-treated animals, the effects on the viscera cannot be accounted for merely by the precocious onset of adult development. As has already been noted, JH applica-

TABLE VI

Sensitivity of early and late prepupae to a single application of a mixture of juvenile hormone analogues

Dose (μg)	Number treated	Number failing to diapause	Juvenile characters in resulting moths†						
			External*	Internal**					
				Thorax	Fat body	Gut	Gonads	Accessory glands	Abdominal intersegmental muscles
Application at time of ocellar retraction (days 4–5)									
5	2	0	0	0	0	0	0	0	0
10	9	3	0	1.8	2.7	2.3§	2	1.7	2
50	12	11	0.4	3.3	4.2	2.9§	3.9	2.6	4.2§
150	4	4	0.4	3.5	4.6	3.2§	4.2	3	4.3§
300	2	2	2	4.5	5	5	5	4.5	5§
Application to white prepupa (days 8–9)									
1	2	0	0	0	0	0	0	0	0
5	6	4	0	1.5	2.8	2	2.2	1.5	0.8
10	5	3	0	1.7	2.0	0.3	2.2	1	0
50	5	4	0	2.5	4.2	3	3.6	2.7	3
100	3	3	0.3	1.8	3.7	2.5	3.3	2	3
150	3	3	0.2	2	3.7	1	2.7	1.7	1.7

† When there is more than one treated individual, an average score is used. This average is based only on the scores for nondiapausing individuals.

* Scoring of external characteristics based on pupal assay for juvenile hormone, Williams (1961).

** Scoring of internal characteristics based on Figure 2.

§ At least one individual showed retention of larval characters.

tion to the early prepupa retards the pupal transformation of the internal organs. Consequently, in response to the secretion of ecdysone immediately after pupal ecdysis, these larval or larval-pupal viscera can only become pupal or pupal-adult.

DISCUSSION

Juvenile hormone and the larval-pupal transformation

The activity of the corpora allata of the Cecropia larva declines throughout the fifth instar and prepupal period and becomes zero by the time of pupal ecdysis (Williams, 1961). The titer of extractable JH from a closely related, nondiapausing species, *Samia cynthia ricini*, also shows these same changes (Patel and Madhavan, 1969). The declining titer of JH is thought to be the reason why pupation rather than another larval molt occurs at the conclusion of the fifth larval stage. Yet, as seen in Tables II and III, neither single nor multiple doses of JH were able to cause a normal fifth instar to molt into a sixth instar larva.

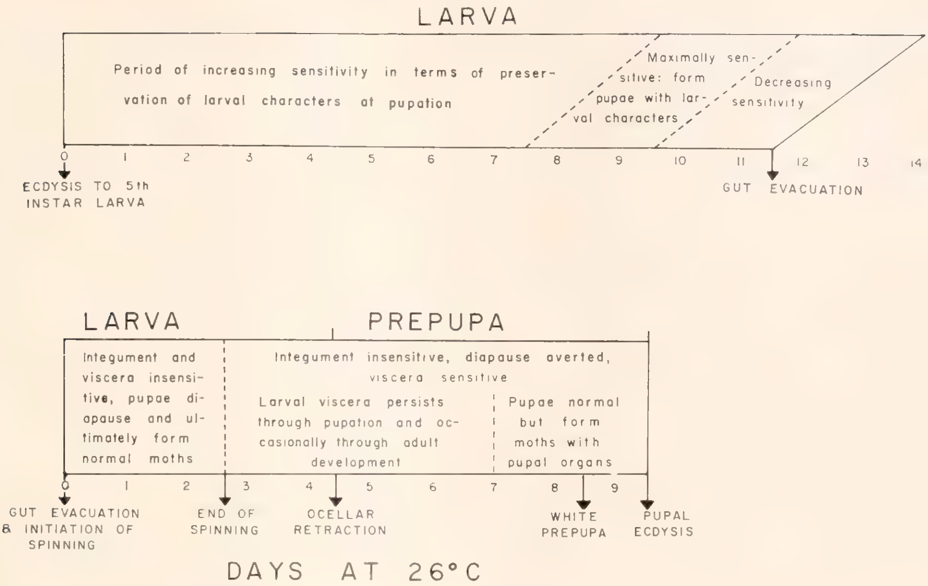
LARVA
Period of increasing sensitivity in terms of preservation of larval characters at pupation
Maximally sensitive: form pupae with larval characters
Decreasing sensitivity
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
ECDYSIS TO 5th INSTAR LARVA
GUT EVACUATION
LARVA
PREPUPA
Integument and viscera insensitive, pupae diapause and ultimately form normal moths
Integument insensitive, diapause averted, viscera sensitive
Larval viscera persists through pupation and occasionally through adult development
Pupae normal but form moths with pupal organs
0
1
2
3
4
5
6
7
8
9
GUT EVACUATION & INITIATION OF SPINNING
END OF SPINNING
OCELLAR RETRACTION
WHITE PREPUPA
PUPAL ECDYSIS
DAYS AT 26°C

FIGURE 3. Time-table of developmental events at 26° C during the fifth larval instar and prepupal stage. The changing sensitivities to topically applied JH are indicated. The slanted lines indicate the variation in the timing of the recorded events in normal, untreated individuals.

On the basis of the experiments reported here, we now see that certain epidermal structures of fifth instar larvae remain sensitive to JH, especially when the latter is applied 4 days before the initiation of spinning. The larval tubercles were found to be most sensitive, followed by the tergal epidermis, the mouthparts, and finally the thoracic legs and abdominal prolegs. Nearly all of these larvae diapaused after pupation; after preliminary chilling they developed into normal adults, except that the tubercles and other sites where larval characters had been retained underwent pupation instead of adult differentiation (Figs. 1A and B). This result is the same as that reported by Willis (1969) for Cecropia infected with *Nosema*.

Figure 3 summarizes the effects of exogenous JH from the outset of the fifth instar until the ecdysis of the pupa 22 to 24 days later. The metamorphosis of the internal organs was unaffected when JH was applied prior to the onset of spinning. By contrast, during the entire prepupal period, JH had pronounced effects on the viscera but little or no effects on the metamorphosis of the epidermis. These findings imply that at the time of spinning the epidermis becomes insensitive to JH, whereas the viscera become sensitive. These results are in marked contrast to those obtained with *Galleria* in which both the integument and the internal organs are sensitive to JH before, but not after spinning (Sehnal, 1968; Sehnal and Meyer, 1968).

The difference between the two species almost certainly hinges on the duration of the pupal stage. *Galleria* initiates adult development within 24 hours after pupal ecdysis; that being so, it is necessary for the internal organs of *Galleria* to synchronize their metamorphosis with that of the integument. By contrast, Cecropia undergoes a pupal diapause which persists for many months. Therefore, the larval-pupal transformation of the internal organs can be delayed or postponed. Schneiderman and Williams (1953) showed that during the first 10 days after pupal ecdysis, the respiration of Cecropia declines to the diapause level. Similarly, in this period RNA synthesis continues in the internal organs as well as in the epidermis which is depositing the entire pupal endocuticle (Berry, Krishnakumaran, Oberlander, and Schneiderman, 1967).

JH and the prevention of diapause

JH applied to prepupae prevents diapause by blocking the normal inactivation of the brain at the time of pupal ecdysis (Williams, 1952). As seen in Table VI, the sensitivity of the brain to JH is maximal at precisely the time when it normally is shut-off. The exogenous JH prevents the complete larval-pupal transformation of the brain. The net result is that the brain retains its ability to secrete the prothoracicotropic hormone (Table V).

JH and the disruption of metamorphosis of the internal organs

As seen in Table IV, the metamorphosis of internal organs was affected by JH application to prepupae, but only when diapause was averted. The larval structures which occasionally persisted throughout metamorphosis were in virtually all cases tissues which normally break down prior to pupal ecdysis. JH apparently blocked this process so that they persisted into the adult.

Those organs which undergo a distinct metamorphosis rather than dissolution seemed to respond to JH by a retardation in metamorphosis rather than a complete cessation of change. Therefore, in treated individuals in which diapause was averted, the initiation of adult development caused those organs which were still larval to become pupal and those which were partway through the larval-pupal transformation to become pupal-adult. Preliminary experiments suggest that when either the gonads or fat body are transplanted from treated individuals just after pupal ecdysis and are caused to develop immediately by implanting them into normal chilled pupae, they transform into pupal or pupal-adult organs just as they do *in situ*. By contrast, when the treated prepupae underwent diapause, apparently the internal organs "caught up" and susequently developed into normal adult organs.

The mechanism of JH action in the larval-pupal transformation

According to Williams (1961), juvenile hormone must be present at the outset of adult development to exert its *status quo* action. When JH injection was delayed until the 3.5 day after apolysis, it had little or no effect on adult development (Williams, 1968). Apparently, the pupal epidermis is most sensitive to JH when it is undergoing the early rounds of DNA synthesis just after apolysis (Bowers and Williams, 1964; Krishnakumaran, Berry, Oberlander, and Schneiderman, 1967; Schneiderman, Krishnakumaran, Bryant, and Sehnal, 1969). Similarly, the final stage larval epidermis of *Galleria* becomes insensitive to JH once the cells have replicated their DNA (Sehnal, 1969; Sehnal and Novak, 1969: Schneiderman *et al.*, 1969).

In Cecropia, Krishnakumaran *et al.* (1967) have shown that DNA synthesis begins at the base of the tubercles on the first day of spinning and in the general epidermis the next day. To exert its *status quo* effect on the integument, JH had to be applied at least one and optimally two to three days before the onset of this DNA synthesis.

By contrast, in the viscera sensitivity to JH persists throughout the prepupal period. Yet only in the brain and hemocytes does DNA synthesis continue until after the pupal ecdysis (Krishnakumaran *et al.*, 1967). In all the other tissues and organs such as the midgut, fat body, and thoracic musculature, DNA synthesis shuts off at least 3 days before the pupal ecdysis although extensive histolysis and reorganization continue. Thus, JH can act on the viscera even after DNA synthesis has ceased, apparently by interfering with degradation and reorganization of the tissues.

I thank Mrs. Su-mei Wang and Miss Saundra Troisi for rearing the Cecropia on the artificial diet; Professor E. J. Corey for providing the *dl-trans, trans, cis* Cecropia hormone; Dr. John Siddall for providing the α-ecdysone; Professor C. M. Williams for providing the synthetic juvenile hormone mixture and helpful discussions during the preparation of this manuscript; and Dr. James Truman for drawing the figures and a critical reading of the manuscript.

SUMMARY

1. Pupae retaining larval integumentary structures were formed after topical applications of Cecropia juvenile hormone or a mixture of juvenile hormone analogues to fifth instar Cecropia larvae prior to the initiation of spinning. Even daily applications of high doses did not result in a supernumerary larval molt.

2. The integument was most sensitive to juvenile hormone (JH) two to four days prior to spinning.

3. During the period of cocoon spinning, both the larval integument and viscera were insensitive to exogenous JH.

4. During the prepupal period, the integument remained insensitive but the larval-pupal transformation of the viscera was retarded by topical application of JH.

5. Juvenile hormone applied during the prepupal period prevented the normal "shutting-off" of the prothoracicotropic activity of the brain prior to pupal ecdysis. Consequently, the pupae did not diapause but initiated adult development within 5 days. The subsequent adults were normal externally but retained pupal or pupal-adult viscera with occasional retention of larval characters.

6. Unlike the epidermis, the viscera are sensitive to JH not only at the reported time of DNA synthesis, but also during the following period of tissue histolysis and reorganization.

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OBSERVATIONS ON THE MORPHOLOGY AND LIFE-HISTORY OF THE DIGENETIC TREMATODE, *LEPOCREADIUM SETIFEROIDES* (MILLER AND NORTHUP, 1926) MARTIN, 1938[1]

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Miller and Northup (1926) reported on a one year survey of trematode. infections in *Nassa obsoleta* (Say), taken at Quisset Harbor, near Woods Hole, Massachusetts. They examined 8,875 snails and described five new species of cercariae. The incidence and intensity of infection of each species were recorded for each month. One of these species, an ophthalmotrichocercous distome larva, was designated *Cercaria setiferoides.* Both redial and cercarial generations were described.

Martin (1938) redescribed the species and conducted experiments to disclose the life-cycle. The cercariae were recognized as members of the genus *Lepocreadium* Stossich, 1904 and the species was named *Lepocreadium setiferoides* (Miller and Northup, 1926).

Peters (1961) studied the development of the excretory system in the cercariae of species in the Lepocreadiidae and Acanthocolpidae. As the representative of lepocreadid cercariae, he selected *L. setiferoides.*

Although there is sufficient general agreement to assure that these three investigations dealt with the same species, there are serious discrepancies in the accounts. The disagreements are concerned with degree of development of the digestive and reproductive systems, the number of paired setigerous tufts on the tail, the number and arrangement of penetration glands and details of the excretory system. During the summers of 1969, 1970 and 1971, study of *L. setiferoides* has been made in an attempt to resolve the differences in previous accounts and provide additional information on the species. This report presents a redescription of the successive stages in the life cycle and the results of experimental infections in disclosing new secondary intermediate hosts. Finally, a reconsideration of the synonymy restores the validity of *Lepocreadium setiferoides* (Miller and Northup, 1926) Martin, 1938.

OBSERVATIONS AND RESULTS

Life cycle and larval stages

The present study of *Cercaria setiferoides* has provided information to clarify and supplement previous descriptions. Both Miller and Northup (1926) and Martin (1938) described rediae of *C. setiferoides*, and their observations have been confirmed. However, neither reported more than one generation of rediae. There are

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at least two generations of rediae. Each daughter redia produces one or more daughters before it begins to produce cercariae.

In living cercariae, twitching of the tail makes counting of the lateral setigerous tufts a frustrating experience, but examination of fixed specimens, killed in hot water, has permitted a certain count; there are 33 pairs and a terminal tuft. The structure of the lateral pairs is similar to that of the "finlets" on the tails of cercariae of *Neopechona pyriforme* as described by Stunkard (1969a). The wall of the tail consists of external circular and inner longitudinal muscles and the central core contains a linear series of large "caudal bodies" or "glycogen cells." The cercariae swim tail first and the action of the paired oar or paddle-like tufts induces rapid progression. The ocelli orient the cercariae with reference to light and swimming movements take them toward the dark side of the container.

The digestive system is well developed. The cercariae emerge from the rediae while immature and complete their development in the haemocoele of the snail. They ingest the snail's blood and their ceca are filled with material that stains intensely with neutral red. When the body is retracted, the material in the ceca is disposed in a spiral coil. The intestinal ceca become narrow posteriorly and communicate with the posterior end of the excretory vesicle, forming a uroproct.

There are eleven pairs of penetration glands; on each side there are three gland cells lateral and posterior to the ocellus; their ducts pass forward lateral to the ocellus. Eight other cells are situated in front of and at the sides of the acetabulum; their ducts pass forward medial to the ocellus. When the anterior end of the cercaria is retracted, certain of the glands nearest the median plane may lie posterior to the digestive ceca, as shown in the figure of Miller and Northup (1926). As a result of pressure from the overlying ceca, the initial portions of the ducts from these cells are empty and not easily observed. There are no cystogenous gland cells and the metacercariae do not encyst.

The reproductive systems are well developed. The testes and ovary are clearly recognizable as described and figured by Martin (1938). When a specimen is retracted, as shown in Martin's figure 3, the testes are opposite but, on extension, pressure of the excretory vesicle causes the testes to be situated obliquely, with the left one slightly anterior to the right. Miller and Northup (1926) described the excretory vesicle and the primary and secondary collecting tubules, but the flame-cell formula was not resolved. Martin (1938) described the sphincter at the posterior end of the bladder, and confirmed the observations on the collecting tubules, but his observations on the pattern and number of flame-cells were admittedly incomplete. Peters (1961) was concerned principally in testing the validity of the taxonomic system proposed by La Rue (1957), which was based primarily on the structure of the excretory bladder and location of the primary excretory pores. The wall of the excretory vesicle is epithelial, composed of cells derived from the mesoderm. In the mature cercaria the primary collecting ducts emerge at the sides of the vesicle and pass laterad along the anterior faces of the testes: then anteriad to the level of the anterior margin of the acetabulum. Here they divide into anterior and posterior secondaries. On each side the anterior branch passes forward to the level of the ocellus where it gives off an anterior tertiary tubule and turns posteriad. The anterior tertiary receives a capillary from a flame-cell located lateral to the ocellus, one from a flame-cell situated immediately anterior to the ocellus, one from a flame-cell that is lateral in position, and the tubule divides to form two capillaries, one leading to a flame cell immediately posterior to the oral sucker and the other lateral to the sucker. The recurrent branch gives off a second tertiary that divides in a similar manner and drains five flame-cells in the post-ocellar region and a third tertiary that drains five flame-cells in the acetabular region. The disposition of the branches and flame-cells in the posterior half of the body is the exact obverse of that in the anterior half. The location and arrangement of the flame-cells are shown in Figure 1, and the flame-cell formula is $2[(5 + 5 + 5) + (5 + 5 + 5)]$.

Descriptions of cercariae often list maximum, minimum and average sizes of living structure. Such measurements are made under coverglass pressure which varies with the amount of water in the preparation. Length and width of any structure increases as the water evaporates and differences in measurements may vary from 10 to 50 per cent. More accurate and precise figures are obtained by killing the cercariae in hot water before fixation. Standard fixing fluids may follow immediately after killing. This procedure gives specimens of uniform size and shape, which provides a standard for comparison with other species. Cercariae killed in whirling hot water give the following measurements in mm: length of body, 0.225; width of body, 0.115; length of tail, 0.650; width of tail at base, 0.040; acetabulum, 0.040; oral sucker, 0.058; pharynx, 0.021; acellus, 0.012 by 0.015; length of setae, 0.07 shorter distally.

Experimental infections

New experimental work falls into two categories. A variety of invertebrates were exposed to cercarial infection, and subsequently specimens of the species of annelid worms and turbellarians successfully invaded by the larvae were fed to the primary host, the winter flounder, *Pseudopleuronectes americanus.*

The cercariae live for about 48 hours at room temperature; older and somewhat exhausted specimens sink to the bottom where they attach by the tips of their tails. Possible secondary intermediate hosts have been exposed in finger-bowls with large numbers of recently emerged cercariae. Obviously, exposure to hundreds or thousands of cercariae in the confines of a finger-bowl is not comparable to conditions in nature, but failure to obtain infection eliminates any species as a possible intermediate host. The invertebrates employed in exposure experiments often harbored metacercariae of natural infections by other species but the conditions of the experiment made it possible to distinguish between natural and experimental infections, especially since *Cercaria setiferoides* does not encyst. No infections were obtained in crustaceans or in either gastropod or bivalve molluscs, but one surprising observation was to find metacercariae of *Zoogonus lasius* encysted in the limpet, *Acmaea intestinalis.* Of the common scyphomedusae. *Chrysaora quinquecirrha,* was the only species invaded. The ctenophore, *Mnemiopsis leidyi,* which harbors metacercariae of other lepocreadid species, was not attacked. Large polychaete annelids of several families, including *Aphrodita hastata, Arabella iricolor, Clymenella torquata, Glycera americana, Glycera dibranchiata, Lepidonotus squamatus, Nephtys bucera, Notomastus latericeus, Pectinaria gouldii, Scoloplus fragilis,* and *Sthenelais boa* were also immune. But the spionids, *Polydora ciliata* and *Polydora ligni* were susceptible and were attacked vigorously (Figure 2). *Cercaria setiferoides* also invaded the acoelous turbellarian, *Childia* sp. The identity of the species of *Childia* in the Woods Hole area is uncertain. Hyman (1959) listed *Childia spinosa* Graff, 1917 and *Childia baltica* Luther, 1912 as synonyms of *Childia groenlandia* (Levinsen, 1879) Meixner, 1925. The polyclad turbellarians, *Euplana gracilis* and *Stylochus ellipticus*, were attacked and metacercariae were distributed in the bodies of the turbellarians. The nature of the secondary intermediate hosts probably explains the observations of Linton (1921) and Martin (1938), that infection occurs only in young and small fishes, since older fishes, six to eight inches in length, feed on other and larger organisms.

Laboratory infected intermediate hosts were fed to a small, one-year old winter flounder, *Pseudopleuronectes americanus*, and the fish was autopsied two weeks later. Twenty juvenile specimens of *L. setiferoides* were recovered from the intestine.

Systematic Review and Discussion

It is now worth briefly reviewing earlier work on the life-cycle and systematics of this and possibly allied forms. Miller and Northup (1926) reported about 30 pairs of setigerous tufts on the tail; Martin (1938) found 35 pairs and a terminal unpaired group. Miller and Northup described a well developed digestive tract with wide intestinal ceca extending to the posterior end of the body; they noted the jelly-like contents of the ceca stained intensely with neutral red. Martin (1938) stated, page 465, "The intestine is rudimentary, sometimes appearing as two strings of cells, staining deeply with neutral red, extending from the esophagus to near the posterior end of the body." Miller and Northup (1926) observed, page 499, "Several cell masses in Anlage of reproductive system; no interpretation ventured as to parts of adult system represented." Martin found, page 465, "The reproductive system is well developed. The two testes have slightly lobed or smooth margins and are located in the posterior one-third of the body. The ovary lies slightly to the right of the mid-line between the anterior testis and the ventral sucker." Miller and Northup (1926) found, page 498, "Eleven pairs of larval glands, preacetabular in position, arranged in three groups; no observable ducts from the most posterior six glands; glands of all groups strongly acidophilic in all combinations of stains; lightly stained with intra-vitam neutral red, but not with toluidine blue." Martin (1938) reported "Eight cephalic glands are present on each side of the body and partially surround the ventral sucker. Their ducts pass anteriorly and open to the exterior at the anterior end of the body. These glands stain deeply with neutral red but in the same general region there appear to be other glands that do not absorb this stain. From ten to fifteen cystogenous glands are scattered irregularly through each half of the body." Miller and Northup (1926) described the excretory vesicle with its concretions and the primary and secondary collecting tubules of the excretory system; they reported a succession of single flagella in the wall of the main lateral tubules, and although many flame-cells were observed, the pattern was not resolved. Martin's account of the excretory system is in general agreement; he found 24 flame-cells on each side of the body which appeared arranged in groups of threes. He reported, "However, one of the capillaries of each group joins the secondary collecting tube independently of the other two."

Peters traced development of the excretory system from the condition in the embryo to the definitive pattern in the mature cercaria. The primary excretory

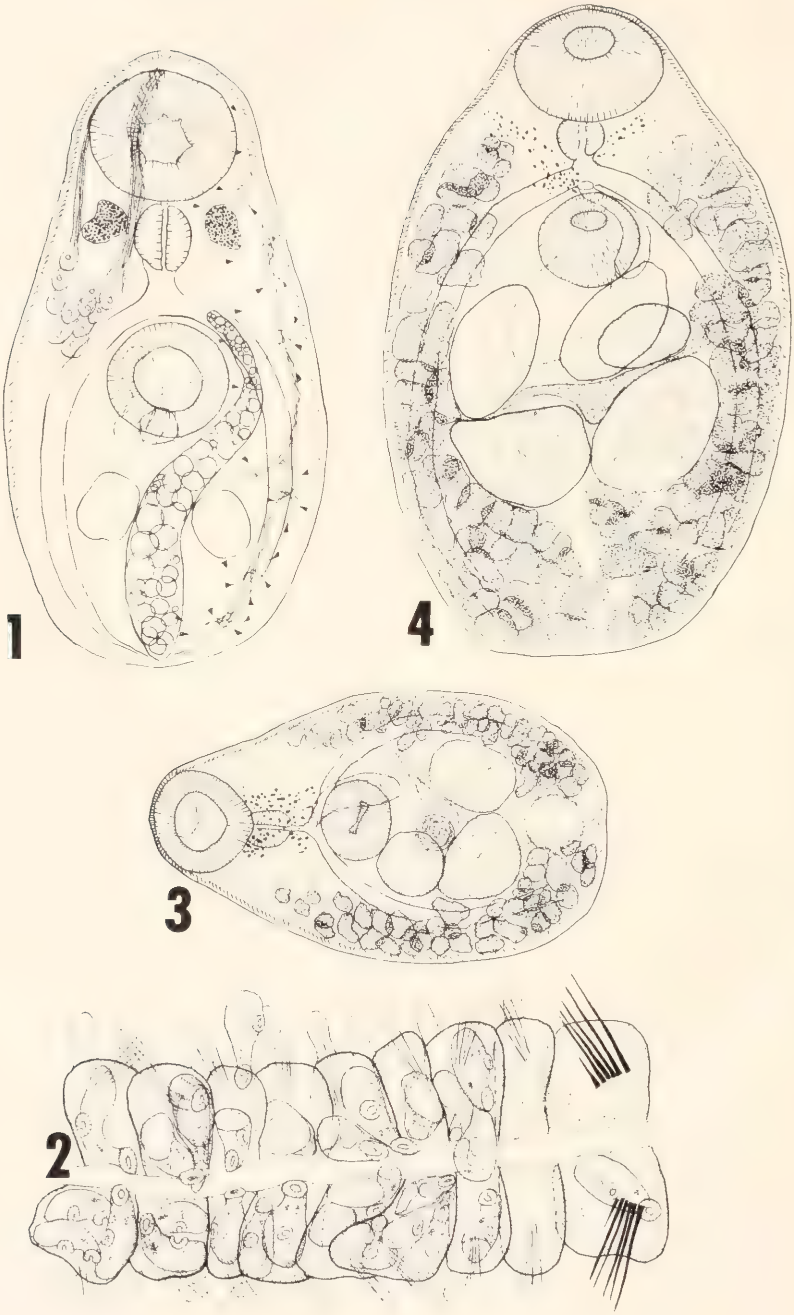

Figure 1. *Cercaria setiferoides,* body, drawn from pencil sketches of living specimens, showing penetration glands, Anlagen of the gonads, the digestive and excretory systems, with location of the flame-cells.

pores were lateral, at the body-tail furrow. The primary ducts fused to form the excretory bladder, which elongated as it acquired an epithelial wall. A sphincter was formed at the posterior end of the bladder, followed by a small atrium at the body-tail junction. The flame-cell formula was given as $2[(3+4+5)+(5+5+5)]$. Peters observed that as the tail grows, a longitudinal row of conspicuous transitory cells appears in the axis of the tail.

Martin isolated the snails in finger-bowls to check on the emergence of cercariae. He found that they emerge both diurnally and nocturnally; that they are photonegative and swim rapidly by lashing of the tail. The cercariae were observed to penetrate and encyst in the triclad turbellarian, *Procerodes warreni*, and in spionid polychaete annelids. The metacercariae did not increase much in size but the reproductive organs became more mature. After an interval of several days, the infected intermediate hosts were fed to small flounders and sand dabs. *Nereis virens* was used as food for the fishes since it was found that this worm would not serve as a second intermediate host of *L. setiferoides*. Martin noted that the experimental hosts may not be the natural ones, particularly *Procerodes*, which is found in rocky regions that are not the habitats of the flatfish. Natural infections were found in most of the small flounders and sand dabs, so that suitable experimental animals, known to be uninfected with this parasite, were not available. However, the small flounders and sand dabs were fed infected *Procerodes* and spionids over a period of about one month and various stages of development from the metacercariae to the adult were recovered. Martin (1938) found these worms in very small flounders but larger fishes, six to eight inches in length were rarely infected. He stated, page 469, "This probably explains the fact that Linton did not find this species in his extensive study of fish parasites." Martin stated that the adult of *L. setiferoides* had not been described previously, but there is evidence that Linton did observe the juvenile worms and that he described the adults under his account of *Lepocreadium trullaforme*.

Linton (1921) reported on the food of young flounders, *Pseudopleuronectes americanus*, collected from 36 stations in the Woods Hole region from May 2 to November 2, 1915 and 1916. A total of 398 fishes were examined; they varied from 25 to 190 mm in length, although most were less than 100 mm long. The contents of the stomachs and intestines were analyzed. The food of the smallest fishes consisted chiefly of copepods; slightly larger fishes took amphipods and in fishes 40 mm long the intestine contained setae of annelids, probably *Polydora* spp., since these are the only species common in the area and small enough to be ingested by fishes of that size. In the stomachs of small fishes, 30 to 40 mm in length, he found large numbers of appendiculate distomes, hemiurid species that use small crustaceans as intermediate hosts, and slightly larger fishes harbored unidentified distomes, apparently juveniles that very well could have included *L. setiferoides*.

FIGURE 2. *Polydora ligni*, showing the modified fifth setiger and eight succeeding somites, with metacercariae of *L. setiferoides*, exposed one day in finger-bowl.

FIGURE 3. *L. setiferoides*, juvenile specimen from *Menticirrhus saxatilis*, collected 21 August, 1918, by E. Linton; on slide No. 8278 in the Helminthological Collection of the U. S. National Museum; same magnification as Figure 4.

FIGURE 4. *L. setiferoides*, mature specimen, 0.46 mm long, on same slide as Number 3; part of a collection of distomes included by Linton (1940) in his description of *Lepocreadium trullaforme* n. sp.

The new observations, presented above, correct the discrepancies and errors in the previous accounts, reveal the existence of at least two generations of rediae, and disclose several new secondary intermediate hosts, both annelid and turbellarian.

In a posthumous publication, Linton (1940) reviewed, revised and supplemented his earlier studies on the trematodes of fishes mainly from the Woods Hole region, Massachusetts. In this paper he described *Lepocreadium trullaforme* n. sp., to contain worms taken at different times from various species of fishes. These included:

(1) two specimens from the American sole, *Achiurus fasciatus*, reported as *Distomum* sp., in Linton (1901, page 487, Fig. 51); the type specimen is in the Helminthological Collection of the U.S. National Museum under the number 8275.

(2) specimens from the long spined sclupin, *Acanthocottus octodecimspinosus*, taken 22 December, 1906; 12 May, 1913; 20 April, 1914; 26 April, 1915; specimen #8276, U. S. N. M., named as type.

(3) one specimen from the kingfish, *Menticirrhus saxatilis*, taken 11 September, 1907; U. S. N. M., #8277.

(4) many specimens, some immature, from young kingfish, *M. saxatilis*, collected 21 August, 1918; U. S. N. M., #8278.

(5) eight specimens from the white perch, *Morone americana*, collected 8 August, 1910; U. S. N. M., #8279.

(6) specimens from the winter flounder, *Pseudopleuronectes americanus*, described as *Distomum areolatum* Rudolphi in Linton (1900, page 293, Figs. 60–63); U. S. N. M., #6517.

(7) specimens from the cunner, *Tautogolabrus adspersus*, identified as *Distomum areolatum* Rudolphi in Linton (1901, page 462).

As noted, Linton (1940) included *Distomum areolatum* Rudolphi of Linton, 1900 in *Lepocreadium trullaforme* and Yamaguti (1958) suppressed *L. trullaforme* as a synonym of *D. areolatum* Rudolphi of Linton, 1900. Stunkard (1969b) redescribed the species, and showed that it is distinct from *Distomum areolatum* Rudolphi, 1809, and transferred it to *Lepocreadium* Stossich, 1904, as *Lepocreadium areolatum* (Linton, 1900) n. comb. Study of the specimens from the Helminthological Collection listed above, shows that with the exception of those under #8278, all are *Lepocreadium areolatum* (Linton, 1900) Stunkard, 1969.

The small specimens listed under #8278, from young *M. saxatilis*, 55 to 110 mm in length, are specifically distinct from the single specimen from the same host, designated #8277 in the Helminthological Collection. There are 30 of these small specimens, mounted on a single slide. The description of these worms as given by Linton (1940) is reasonably complete. He gave the size, shape, sizes of suckers and gonads, and disposition of the vitellaria. Two specimens are represented in his Figures 55 and 56. As characteristic features of the species he noted the large size and small number of eggs. He reported that no more than two eggs were present in any worm. Some of the small worms were immature and contained no eggs but eggs were present in very small specimens. The eggs measured 0.10 by 0.06 mm and were partially collapsed.

The specimens have now been restudied. One of the largest of them is shown in Figure 4. It is 0.46 mm in length and is one of two that contains three eggs; all the other gravid worms have only one or two eggs. The largest juvenile specimen is shown in Figure 3. In it the seminal receptacle is filled with spermatozoa and other specimens of approximately the same size contain a single egg. The small size, large size of eggs, and over-all morphology of these worms are so strikingly similar to the adults of *L. setiferoides* that the identity is apparent. A detailed comparison with the descriptions of Martin and Linton confirm the allocation.

The genus *Lepocreadium* was erected by Stossich (1904) with *Distomum album* Stossich, 1900 as type species. Palombi (1937) reported that *Cercaria setifera* Monticelli, 1914, *nec* Joh. Müller, 1786, is the larva of *L. album*. The life-cycle of a second species, *Lepocreadium pegorchis*, was reported by Bartoli (1967). Many species with divergent morphology have been assigned to *Lepocreadium* and as a result the generic concept has become indefinite and uncertain. Sogandares-Bernal and Hutton (1960) reported on marine species from the North American Atlantic. They stated that the status of these species has been in a state of confusion for the last twenty years. They declared that *L. truttaforme* Linton, 1940 was a composite of at least two different species and redescribed the type specimen from the sculpin, *Acanthocottus octodecimspinosus*, #8276 in the Helminthological Collection of the U. S. National Museum. *Lepocreadium retusum* Linton, 1940 was transferred to *Neolepidapedon*. Two new species, *Lepocreadium caballeroi* and *Lepocreadium opsanusi*, were described. *Lepocreadium caballeroi*, based on a single specimen from *Menticirrhus saxatilis*, is probably identical with *L. areolatum* (Linton, 1900) from the same host and *L. opsanusi* is very similar to and may be identical with *Lepocreadium trulla* (Linton, 1907) Linton, 1910. *Distomum* sp. of Linton (1901, page 458, Fig. 346) from the scup, *Stenotomus chrysops* at Woods Hole was tentatively named *Lepocreadium ovalis* n. sp. by Manter (1931), based on a variety of specimens from the pinfish, *Lagodon rhomboides*, taken at Beaufort, North Carolina. Yamaguti (1958) listed the species as *L. ovale* Manter, 1932 but its validity is suspect. Linton (1940) included *Distomum pyriforme* Linton, 1900 from *Palinurichthys perciformis*, a species he (1901, 1905) reported from other fishes, as a member of *Lepocreadium*. Stunkard (1969a) noted that the specimens listed as *L. pyriforme* by Linton (1940) comprised a heterogenous collection of different species but did not include *Distomum pyriforme* Linton, 1900. He worked out the life-cycle of the last species and showed that it could not be included in the genus *Lepocreadium*. Instead, it was named type of a new genus, *Neopechona*.

SUMMARY

Cercaria setiferoides, an ophthalmotrichocercous distome larva from *Nassa obsoleta*, was described by Miller and Northup (1926). Its life-cycle was reported by Martin (1938) and the excretory system was studied by Peters (1961). Martin found the larvae encysted as metacercariae in the turbellarian, *Procerodes warreni*, and in the annelid, *Spio* sp. The final hosts were small flounders and sand dabs. There are serious disagreements and discrepancies in the three accounts and a redescription of the successive stages in the life-cycle provides new information as well as the correction of previous errors. Experimental infections have disclosed new secondary intermediate hosts and the adult stage is regarded as identical with worms described by Linton (1940) as *Lepocreadium trullaforme* n. sp.

But *L. trullaforme* was suppressed by Yamaguti (1958) as a synonym of *Distomum areolatum* Rudolphi of Linton, 1900, and Stunkard (1969b) redescribed that species as *Lepocreadium areolatum* (Linton, 1900) n. comb. This taxonomic disposition restores the validity of *Lepocreadium setiferoides* (Miller and Northup, 1926) Martin, 1938.

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SYMBIOTIC CHLOROPLASTS; THEIR PHOTOSYNTHETIC PRODUCTS AND CONTRIBUTION TO MUCUS SYNTHESIS IN TWO MARINE SLUGS

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Chloroplasts occur within the cells of the digestive diverticulum of some sacoglossan gastropod molluscs (Kawaguti and Yamasu, 1965; Taylor, 1967, 1968, 1970; Trench, 1969; Trench, Greene and Bystrom, 1969; Greene, 1969, 1970). These chloroplasts, derived from certain marine algae (Siphonales) produce oxygen in the light and fix much more ^{14}C in the light than in the dark (Trench, 1969; Trench *et al.*, 1969; Greene, 1969; 1970; Taylor, 1971b).

It has been shown by radioautography that in the sacoglossans *Tridachia crispata* and *Tridachiella diomedea*, photosynthetically fixed ^{14}C moves from the chloroplasts to various chloroplast-free animal organs, especially the pedal mucus gland. This gland secretes a carpet of mucus over which the slugs crawl (Trench, 1969; Trench *et al.*, 1969).

Recently, Trench, Trench and Muscatine (1970) showed that after specimens of *Placobranchus ianthobapsus* (Gould) were incubated in $NaH^{14}CO_3$ in the light, the mucus secreted by the animals contained ^{14}C-galactose and ^{14}C-glucose. They suggested that chloroplast photosynthesis provided some of the substrates used by the animal in the synthesis of mucopolysaccharides.

To gain insight into the way sacoglossans utilize the products of chloroplast photosynthesis it is necessary first to identify these products in the animal hosts, and then to follow their incorporation into animal substrates. In addition, by comparing the products of symbiotic chloroplasts in their animal hosts with those of intact siphonous algae, possible modification in chloroplast function in the animal cell milieu may be detected.

This paper compares the products of photosynthetic ^{14}C fixation by chloroplasts in the slugs *Tridachia crispata* (Mörch) and *Tridachiella diomedea* (Bergh) with those of chloroplasts in the siphonous marine alga *Caulerpa sertularioides*. The incorporation of chloroplast products into animal mucus is described in detail and the turnover of ^{14}C in the pedal mucus gland of *Tridachia* is estimated.

MATERIALS AND METHODS

(1.) *Collection and maintenance of specimens*

Tridachia crispata and *Caulerpa sertulariodes* were collected from the reefs off Port Royal and Discovery Bay, Jamaica, kept in the shade (about 900–1200 foot candles) in plastic tanks and used within a few days of collection.

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Tridachiella diomedea was collected from mangrove lagoons in the vicinity of La Paz, Baja California, Mexico. Experiments were conducted on board the R. V. DOLPHIN the same day.

(2.) *Experiments*

(a) *Incubations in* $NaH^{14}CO_3$. Whole animals and pieces of alga (2–3 cm) were separately incubated in sea water to which $NaH^{14}CO_3$ was added to give initial activities of 10–25 μc ml. Specimens of *Tridachiella diomedea* were incubated in sunlight (1000–1500 foot candles in the shade) at a temperature of 21° C ± 2° C.

Incubations of *Tridachia crispata* and *Caulerpa sertularioides* were carried out under ambient light conditions (900–1700 foot candles) or under constant intensity light conditions of 900 foot candles delivered by photoflood lamps. Temperature was maintained at 25° C ± 1° C.

(b) *Incubations in* ^{3}*H-leucine*. Whole specimens of *Tridachia crispata* were incubated under conditions similar to those described above for ^{14}C in sea water to which ^{3}H-leucine (100–250 mc/mM, Amersham, England) was added to give initial activities of 10–20 μc/ml.

(3.) *Analytical procedures*

(a) *Chemical fractionation of* ^{14}C*-compounds from animal and algal tissues after photosynthetic* $^{14}CO_2$ *fixation*. After incubation in $NaH^{14}CO_3$, both plant and animal material were extracted under reflux in methanol-chloroform (1:2 v/v) for two hours. This fraction was then extracted with diethyl ether in a separatory funnel. Phase separation was effected by the addition of small quantities of 15% aqueous NaCl. The ether phase was washed with several small aliquots of distilled water, and 100 μl was removed for counting. This extracted lipid fraction was deacylated and the products identified as described previously (Trench, 1971a, 1971b).

After withdrawing 100 μl of the reconstituted aqueous methanol fraction for counting, the remainder was evaporated to dryness under reduced pressure at 40° C and radioactive compounds were recovered in absolute ethanol or dry pyridine. This fraction is referred to as the "intermediary metabolites" fraction. The efficiency of recovery of ^{14}C was 85–95%.

Tissues insoluble in methanol-chloroform were hydrolyzed in 3 M KOH in 80% ethanol. On neutralizing the solution, the precipitate formed was collected by centrifugation. An aliquot of 100 μl of the supernatant, the "protein" fraction, was withdrawn for counting, and the remainder was stored for subsequent analysis.

The alkali insoluble precipitate was treated with 1 M HCl at 100° C for three hours. When neutralized, all the ^{14}C in this fraction from animal tissues was reprecipitated. By contrast, algal tissues were completely solubilized by this treatment. An aliquot of 100 μl of algal tissue hydrolysate ("polysaccharide" fraction) was removed for counting. The remainder was stored for subsequent analysis.

The 1 M HCl insoluble fraction from animal tissues was hydrolyzed in 3 M HCl for 4 hours at 100° C. The solution was neutralized and yielded an insoluble substance which did not dissolve in hot 5% TCA or hot formic acid. This fraction which contained less than 3% of the total fixed ^{14}C was not analyzed further. The insoluble substance was removed by centrifugation and an aliquot of 100 μl of the supernatant (animal "polysaccharide") was collected for counting. The remainder of the samples was stored for subsequent analysis by paper chromatography.

(b) *Paper chromatography.* Solvent systems used during this study were (i) phenol; water (100:40 w/v) for the first dimension and n-butanol: propionic acid:water (142:71:100 v/v) for the second dimension (Bassham and Calvin, 1957) for two dimensional chromatography on Whatman Nos. 1 and 4 papers; (ii) methyl-ethyl-ketone:pyridine:water:acetic acid (70:15:15:2 v/v), (von Holt and von Holt, 1968b); (iii) n-propanol:ethyl acetate:borated water (7:2:1 v/v) and (iv) pyridine:water:ethyl acetate:acetic acid (5:3:5:1 v/v), (Block, Durrum and Zweig, 1958); the latter three systems being used in one dimensional descending chromatography on Whatman No. 1 paper.

Radioactive compounds on developed chromatograms were located by radioautography with Kodak x-ray film and by automatic scanning of one dimensional chromatograms (Loughman and Martin, 1957). Unknown radioactive compounds were identified by "finger-printing" co-chromatography in at least two different solvent systems.

Sugars used in co-chromatography or those produced by hydrolysis of mucus were detected chemically with silver nitrate, p-anisidine hydrochloride or aniline phthalate; amino sugars by the Elson-Morgan reaction (Neuberger and Marshall, 1966).

(c) *Gas-liquid chromatography* (GLC). Samples of mucus secreted by *Tridachia crispata* were hydolyzed in 3 M HCl for 4 hours at 100°C. The hydrolysates were evaporated to dryness and the trimethyl-silyl derivative produced by adding 0.2 ml bis (trimethyl silyl) acetamide to the dried sample and allowing it to react for 24 hours at 20° C. Carbohydrates were separated with 2% SE52 on Diatomite C in a Pye 104 dual FID gas chromatograph, coupled to a Servoscribe recorder. Peaks were identified by use of internal standards.

(d) *Collection of mucus.* Mucus was collected from animals by placing individuals in about 2 ml sea water in a beaker and gently swirling the beaker. This caused the secretion over a period of about 10–15 minutes of copious quantities of mucus. After removal of the animal the mucus was precipitated from solution by the addition of an equal volume of absolute ethanol. After cooling to 3° C, the mucus was sedimented by centrifugation.

(e) *Mucus hydrolysis.* Samples of mucus secreted by *Tridachia* and *Tridachiella* were hydrolyzed in 3 M KOH at 100° C for 4 hours, and the solution neutralized. The unhydrolyzed polysaccharide was reprecipitated by the addition of absolute ethanol to the neutralized solution at 3° C, and collected by centrifugation. After several washes with cold 80% ethanol, the polysaccharide was hydrolyzed with 3 M HCl for 4 hours at 100° C. After hydrolysis, this solution was also neutralized. The products of hydrolysis were recovered in absolute ethanol or pyridine for analysis by paper chromatography.

Secreted ^{14}C-mucus was precipitated as described above (3d) and hydrolyzed in sealed glass tubes in 3 M HCl at 100° C for 4 hours. The HCI was removed and the solutions were concentrated under vacuum prior to analysis by paper chromatography.

(f) *Estimation of total carbohydrate and sulphate in mucus.* The carbohydrate content of mucus was estimated using the phenol-sulphuric acid method of Dubois, Gillies, Hamilton, Rebers and Smith (1956). Colorimetric measurements were made on a Bausch and Lomb Spectronic-20 spectrophotometer at 490 nm.

The sulphate content of mucus was determined by the method of Gibbons and Wolfrom (1962). Sedimented mucus was dried *in vacuo* over P_2O_5 to constant weight.

(g) *Measurement of radioactivity.* Radioactive samples were assayed on planchets with a thin end window Geiger-Müller counter. Corrections were made for self-absorption and background.

(h) *Tissue radioautography.* Animals incubated in $NaH^{14}CO_3$ or ^3H-leucine were prepared for radioautography as previously described (Trench *et al.*, 1969). Paraffin-embedded tissues were sectioned at 5 μm, and were exposed to Kodak AR-10 stripping film at −5° C for 14 to 28 days. Radioautographs were developed as advised by the film manufacturer.

RESULTS

(1.) *Distribution of fixed ^{14}C in* Tridachia, Tridachiella *and* Caulerpa

After six hours photosynthesis in $NaH^{14}CO_3$ the distribution of ^{14}C in organic compounds in the two animals was very similar, while both were distinct from the pattern observed in the alga (Table I). Because of the relatively long incubation period, it was assumed that the pattern of distribution of ^{14}C in animal and algal tissues approached a "steady state" distribution. Further examination of the composition of these fractions was carried out by paper chromatography.

TABLE I

Percentage distribution of ^{14}C in Tridachia crispata, Tridachiella diomedea *and* Caulerpa sertularioides *after six hours incubation in $NaH^{14}CO_3$ in the light. (10 μc/ml $NaH^{14}CO_3$; 900–1000 foot candles)*

	Lipid	Intermediary metabolites	Protein	Polysaccharide
Tridachia crispata	10 (8–12)	45 (41–47)	9 (9–10)	36 (34–43)
Tridachiella diomedea	12 (9–13)	40 (37–43)	8 (7–9)	40 (37–43)
Caulerpa sertulariodes	28 (26–31)	10 (9–15)	10 (9–11)	52 (47–58)

Values represent averages of four experiments (range in parenthesis). Percentages in animals do not include ^{14}C in mucus secreted during the incubation period.

(a) *Lipids.* Lipids extracted from the animals yielded, after deacylation, many labelled compounds, among which occurred glycerol and traces of monogalactosyl-glycerol and digalactosyl-glycerol. These same gylcolipids were also found after deacylation of lipids from *Caulerpa* but relatively much more ^{14}C was found in mono- and digalactosyl-gylcerol (Table II). It seems reasonable to conclude from these comparisons that the glycolipids are characteristic of plant lipids (see also Thompson, 1965; Nichols, 1970) and that those detected in the animals were probably derived from symbiotic chloroplasts. If this interpretation is accurate, it would suggest that symbiotic chloroplasts only synthesize glycolipid to a limited extent.

(b) *Intermediary metabolites.* A wide range of small molecular weight compounds incorporated ^{14}C in the animals and in the alga, the majority of which were distributed amongst the sugar phosphates, amino and carboxylic acids (Table III). Little can be concluded from these data other than the fact that ^{14}C was found mostly in glutamic acid, alanine and succinic acids in both the animals and the plant. The extent to which this may represent dark fixation was not investigated, but dark fixation represents only 4–5% of photosynthetic fixation (Trench *et al.*, 1969). The remainder of the ^{14}C in this fraction was found principally in carbohydrates, as ^{14}C-galactose and ^{14}C-glucose in the animals and ^{14}C-sucrose and ^{14}C-glucose in the alga.

(c) *Protein.* Relatively small amounts of ^{14}C were detected in animal and plant protein (of Table I). Hyrolysis of animal protein yielded mostly ^{14}C-glutamic acid and ^{14}C-leucine. Algal protein hydrolysates were not analyzed.

(d) *Polysaccharide.* When this fraction of animal tissue was hydrolyzed in 3 M HCl, ^{14}C was found mainly in glucose, galactose, and an unidentified pentose in the ratios of 6:1:2 in *Tridachia* and 1:7:4 in *Tridachiella.* This fraction is of interest in that it may represent synthesized but unsecreted mucus. Hydrolysis of *Caulerpa* polysaccharide yielded ^{14}C-glucose, ^{14}C-galactose and ^{14}C-mannose in the ratio 1:5:3.

These results imply that the fate of photosynthetically fixed ^{14}C in the animals with symbiotic chloroplasts is distinct from that in the alga from which the chloroplasts are derived. This may indicate modification of function by chloroplasts in the animals as well as differences in the utilization of products of choroplast photosynthesis by animal and plant tissues.

TABLE II

Percentage distribution of ^{14}C in products of deacylated lipids in Tridachia crispata, Tridachiella diomedea *and* Caulerpa sertularioides *after photosynthetic ^{14}C fixation. Experimental conditions and numbers of replications as in Table I.*

	Mono-galactosyl-glycerol	Digalactosyl glycerol	Sulpholipid (?)	Glycerol	Unidentified and undeacylated lipid
Tridachia crispata	3	2	15	45	35
Tridachiella diomedea	5	2	10	43	35
Caulerpa sertularioides	16	19	7	3	55

(2.) *Incorporation of products of chloroplast photosynthesis into animal mucus*

(a) *Analysis of mucus secreted by* Tridachia *and* Tridachiella. Under experimental conditions, green specimens of *Tridachia* containing chloroplasts secreted 300–400 μg/g (fresh weight)/hr of mucus (expressed as glucose equivalents), while white animals (presumed to be chlorophyll free) secreted about 100–150 μg/g/hr.

Mucus from *Tridachia* contained 125–133 μg $SO_4^{=}$/mg dry weight of mucus, and that from *Tridachiella* contained 40–54 μg $SO_4^{=}$/mg dry weight mucus. It is not known whether sulphate is present as N-sulphate or O-sulphate or both.

Analysis of hydrolysates of mucus secreted by *Tridachia* by paper chromatography and GLC showed glucose, galactose, glucosamine, galactosamine and an unidentified pentose to be present, while hydrolysates of mucus secreted by *Tridachiella* contained glucose, galactose, possible traces of glucosamine and an unidentified pentose as detected by paper chromatography.

Alkaline hydrolysis of mucus from *Tridachia* and *Tridachiella* yielded a series of ninhydrin positive compounds. Although none of these were rigorously identified, compounds behaving chromatographically like leucine and glutamic acid were recognized. It is suggested that these amino acids were derived from the protein associated with the polysaccharide polymer, since it is known that many invertebrate mucins are associated with protein (Hunt, 1970).

(b) *Analysis of ^{14}C in secreted mucus.* Figure 1 shows the ^{14}C-labelled sugars in acid hydrolysates of mucus secreted by *Tridachia* and *Tridachiella* under similar experimental conditions. Most of the sugars detected in unlabelled mucus are represented, but the distribution of ^{14}C in these moieties is different in the two species of slugs (Table IV). In *Tridachia* most of the ^{14}C in mucus is in the glucose moiety, while in mucus from *Tridachiella*, galactose contained most of the ^{14}C, but a smaller amount of ^{14}C was also incorporated into glucose. Taking into account the data on ^{14}C fixed into carbohydrates (Table III) it is suggested that the labeled moieties detected in mucus were derived from chloroplast photosynthesis. Recalling from Tables III and IV that in *Tridachia* the major soluble labelled sugar is galactose, while the chief labelled component of mucus is glucose, it is tempting to suggest an interconversions of sugars before or during mucus synthesis. Such interconversions may have been less complete in *Tridachiella*, since galactose is the chief constituent of both the soluble sugar pool and the mucus sugar moieties. Either

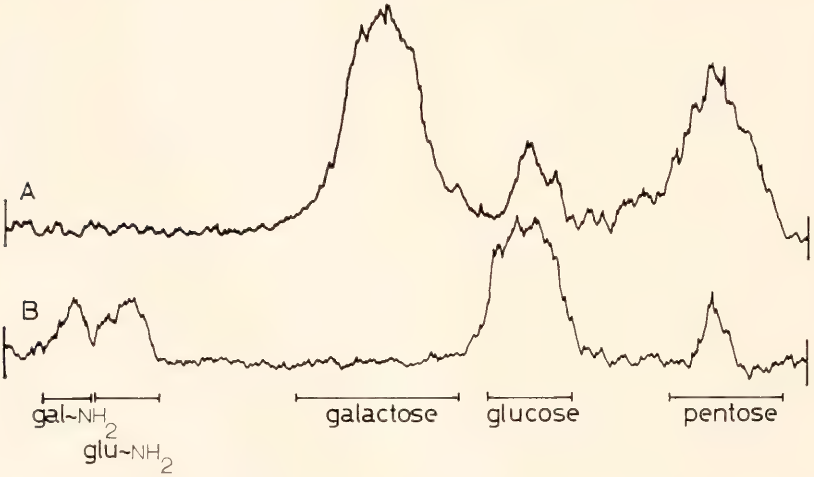

FIGURE 1. Chromatogram scans of ^{14}C-sugars produced by acid hydrolysis of ^{14}C-mucus secreted by *Tridachiella diomedea* (A) and *Tridachia crispata* (B) during incubation in NaH^{14}CO$_3$; solvent system, methyl-ethyl-ketone : pyridine : water : acetic acid, (70 : 15 : 15 : 2, v/v), 36 hours on Whatman No. 1 paper; gal-NH$_2$, galactosamine; glu-NH$_2$, glucosamine.

TABLE III

Percentage distribution of ^{14}C in the "intermediary metabolites" fraction from Tridachia crispata, Tridachiella diomedea *and* Caulerpa sertularioides *after photosynthetic ^{14}C fixation. Experimental conditions and numbers of replications as in Table I*

	Glucose	Galactose	Sucrose	Sugar phosphates and amino acids	Carboxylic acids
Tridachia crispata	5	40	0	39	16
Tridachiella diomedea	9	27	0	48	16
Caulerpa sertularioides	13	0	29	30	28

The values given represent ^{14}C in the different compounds as estimated by scanning the developed radiochromatograms.

the two species of slugs have different modes of mucus synthesis, or the same mode which has been observed at different stages of a complex process.

(c) *Redistribution of ^{14}C in* Tridachia *after a pulse of $NaH^{14}CO_3$.* To obtain further information on the possibility of the interconversion of sugars in mucus synthesis, a series of pulse experiments were carried out on *Tridachia.* Specimens were incubated in $NaH^{14}CO_3$ in the light for 60 min and then transferred to unlabelled sea water for up to 24 hr. Animals were removed at 1, 3, 6, 9, 12 and 24 hr after the end of the pulse. At each sampling, mucus was collected from the sampled animal which was then killed in methanol-chloroform for subsequent chemical analysis.

Analysis of animals by chemical fractionation showed that immediately after the pulse, most of the ^{14}C in the "intermediary metabolites" fraction was in galactose. This is in agreement with the data in Table III. Over the next 24 hr there was a gradual decline in the relative amount of ^{14}C-galactose with a concomitant increase in ^{14}C-glucose, which reached a maximum shortly before the maximum specific activity of secreted ^{14}C-mucus was attained (Fig. 2).

These data strongly support the concept that in *Tridachia,* ^{14}C fixed by the chloroplasts is rapdily converted to galactose which is then available for conversion to glucose, presumably at or near the site of mucus synthesis. It is then possible to infer that in *Tridachiella,* less galactose is converted to glucose, and instead, galactose is incorporated directly into mucus.

(3.) *Rate of turnover of ^{14}C and ^{3}H-leucine in the pedal gland of* Tridachia

(a) *Turnover of ^{14}C.* To gain information on the rate of turnover of chloroplast products in the pedal gland of *Tridachia,* specimens were incubated in $NaH^{14}CO_3$

TABLE IV

Percentage distribution of ^{14}C in mucus secreted by Tridachia crispata *and* Tridachiella diomedea *during incubation in $NaH^{14}CO_3$ in the light*

	Glucose	Glucosamine	Galactose	Galactosamine	5-C sugar
Tridachia crispata	53	24	0	12	11
Tridachiella diomedea	8	0	56	0	36

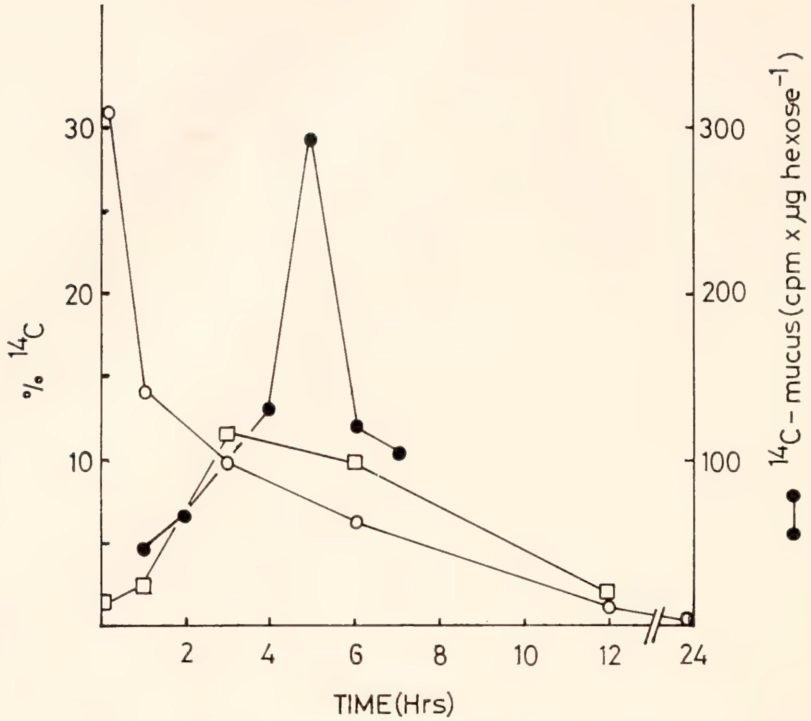

FIGURE 2. Relationship in *Tridachia crispata* between ^{14}C-galactose, ^{14}C-glucose and ^{14}C-mucus after one hour "pulse" label in NaH^{14}CO$_3$ in the light. Labelled glucose and galactose were extracted from the animals, while ^{14}C-mucus was collected from that secreted by the animals; ○———○, galactose; □———□, glucose; ●———●, secreted mucus.

in the light for 1 hr and then transferred to unlabelled sea water. Secreted mucus was collected at hourly intervals beginning immediately after removal of the specimen from the labelled medium, and the specific activity of the ^{14}C-mucus determined. Animals were also fixed in Bouins fluid for subsequent analysis by radioautography.

Figure 3 shows that the specific activity of ^{14}C-mucus reaches a maximum at about 5 hr and then declines, suggesting a turnover time for ^{14}C of about 10 hours.

When the radioautographs were analyzed, an increasing density of silver grains was observed over the pedal gland up to about 6 hr after the pulse (see also Trench *et al.*, 1969), but very few grains were detected after 12 hr presumably because the labelled mucus had been secreted.

These observations are consistent with the estimate of a 10–12 hour turnover time for carbon in the mucus secreting pedal gland of *Tridachia* under these experimental conditions.

(b) *Turnover of* ^{3}*H-leucine.* In view of the observation that the polysaccharide secreted by *Tridachia crispata* had associated protein, it was of interest to ascertain whether exogenously supplied amino acids could be incorporated into mucus-secreting tissues. Specimens were therefore incubated in ^{3}H-leucine for 15

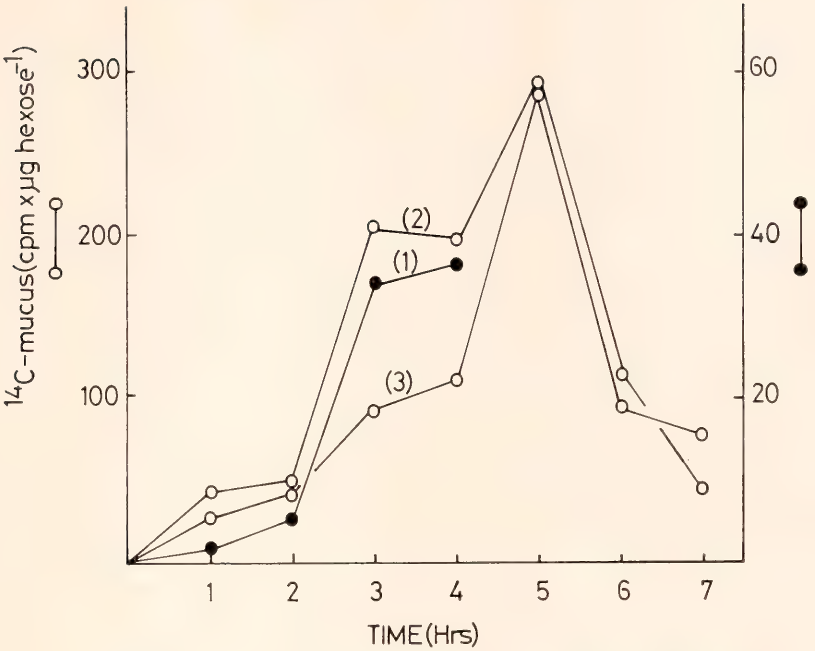

FIGURE 3. Specific activity of ^{14}C-mucus secreted by *Tridachia crispata* after one hour pulse label in $NaH^{14}CO_3$. Experiment (1) was conducted with ^{14}C at a concentration of 10 µc/ml; experiments (2) and (3) at 50 µc/ml.

minutes, and then transferred to unlabeled sea water. Specimens were fixed in Bouin's fluid after 1, 3, 6 and 12 hours in the unlabelled sea water, and the location of radioactivity determined by radioautography.

After 15 minutes in ^{3}H-leucine, most of the insoluble radioactivity in *Tridachia* was associated with the epidermis. After one hour in unlabelled sea water, radioactivity was detected in the end-bulbs of the tubules of the digestive diverticulum and in some (autochromatic) epidermal mucus glands. This contrasts with previous observations which showed that photosynthetic ^{14}C from the chloroplasts was not incorporated into the epidermal mucus glands (Trench, unpublished). Metachromatic mucus cells did not incorporate high levels of radioactivity. Digestive tract tissue also incorporated ^{3}H as did the pedal mucus gland.

After six hours most of the ^{3}H was absent from the epidermal mucus cells, but ^{3}H could still be demonstrated in the pedal mucus gland. After 12 hours however, the pedal gland contained only very little ^{3}H, and the only remaining areas of relatively high activity were the end bulbs of the tubules of the digestive diverticulum.

These observations suggest (a) that the incorporation of amino acids into a polysaccharide-protein complex probably occurs in the pedal mucus gland of *Triadachia*, (b) that leucine may be an important constituent amino acid of the mucus secreted by the pedal mucus gland as well as the epidermal mucus and (c) that the rate of diappearance of ^{3}H from the pedal gland after a pulse "pulse" incorpoation is similar to that of ^{14}C.

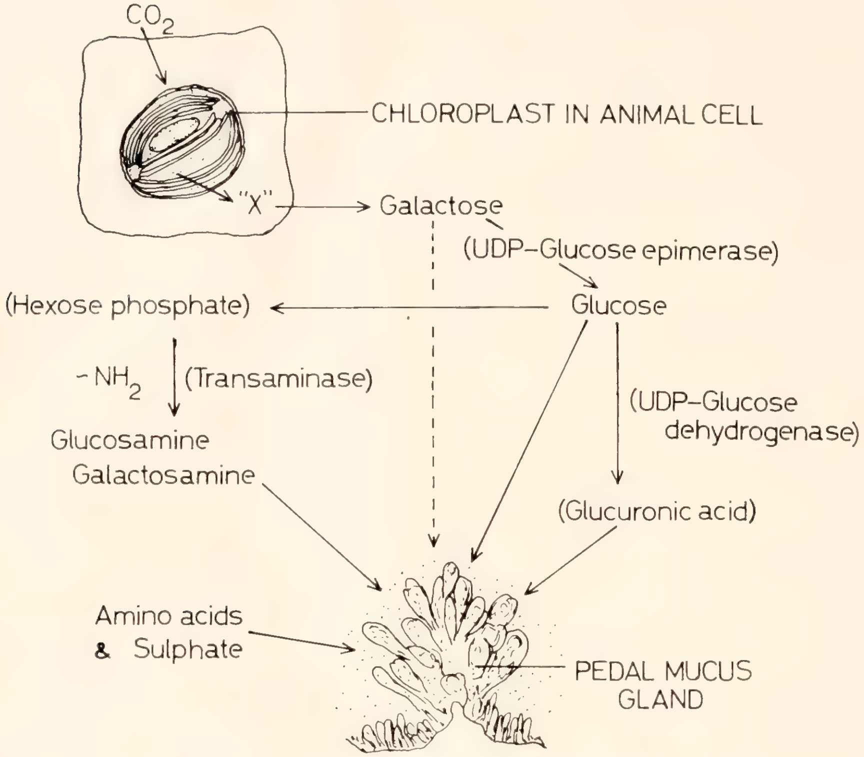
CO_2
CHLOROPLAST IN ANIMAL CELL
"X"
Galactose
(UDP–Glucose epimerase)
(Hexose phosphate)
Glucose
$-NH_2$
(Transaminase)
Glucosamine
Galactosamine
(UDP–Glucose dehydrogenase)
(Glucuronic acid)
Amino acids
& Sulphate
PEDAL MUCUS GLAND

FIGURE 4. Schematic representation of a hypothetical pathway of carbohydrate moving between symbiotic chloroplasts and the pedal mucus gland of *Tridachia crispata*. Enzymes or intermediates in parenthesis have not been identified in the animals studied. Pathways indicated by dotted lines represent alternative possibilities as indicated by data from *Tridachiella* and *Placobranchus*.

DISCUSSION

The distribution of photosynthetically fixed ^{14}C in *Tridachia, Tridachiella* and *Caulerpa* shows marked differences. There could be two reasons for this observation. First, the metabolism of the chloroplasts could become modified in symbiosis and secondly, fixed ^{14}C released by symbiotic chloroplasts may be converted to animal products which are different from those of the plant.

Modification of chloroplast metabolism in animal hosts is suggested by the observation that, in the animals, only trace quantities of ^{14}C are detected in monogalactosyl and digalactosyl digylcerides, while in *Caulerpa*, a larger proportion of fixed ^{14}C incorporated into lipid is found in the galactolipids. This may indicate that less chloroplast membrane synthesis occurs in the slugs over a six hour period than in the plant. This interpretation would be consistent with the reduced chlorophyll synthesis by chloroplasts in slugs observed by Trench and Smith (1970) if the assumption that chloroplast growth and replication is directly correlated with chlorophyll and galactolipid synthesis is tenable (see Boasson and Laetsch, 1969; Constantopoulos, 1970).

By contrast, the difference in metabolism of ^{14}C-sugars between *Caulerpa* and the two slugs is more difficult to explain on the basis of modification of chloroplast behavior. Although ^{14}C-sucrose was found in the plant but not in the animals, it is not known whether this compound was synthesized in the plant cytoplasm or in the chloroplast. Previous studies have suggested that isolated chloroplasts synthesize sucrose (Tolbert and Zill, 1954; Gee, Joshi, Bils and Saltmann, 1965; Shephard, Levin and Bidwell, 1968; Bidwell, Levin and Shepard, 1970) while others maintain that the cytoplasmic fraction is essential (Walker, 1967). The absence of sucrose in the tissues of the slugs is consistent with the interpretation that sucrose synthetase is a cytoplasmic enzyme in the plant, but there is the possibility that the animal cells could convert sucrose released by the chloroplasts to animal sugars, or directly utilize the precursors of sucrose released by the symbiotic chloroplasts.

In the slugs, ^{14}C-galactose and ^{14}C-glucose were the major free sugars detected after fixation of $^{14}CO_2$; but whether these are located in the chloroplasts or in the animal tissues is unclear. The presence of free ^{14}C-glucose and ^{14}C-galactose in slugs with symbiotic chloroplasts after photosynthetic $^{14}CO_2$ fixation appears to be widespread. Greene and Muscatine (in press) also found these labelled sugars in ethanol extracts of the slugs *Elysia hedgepethi* and *Placobranchus ianthobapus* after 2.5 hours photosynthesis. The chloroplasts in these slugs are also derived from a siphonous alga. Comparative studies with chloroplasts isolated from the plant and from animals are currently being undertaken in the hope of clarifying the problem of distinguishing between substances synthesized and retained by the chloroplasts and those released and metabolized by the cell cytoplasmic enzyme systems.

The difference of incorporation of ^{14}C into insoluble compounds in the animals and the alga is also striking. Plant material was readily hydrolyzed in 1 M HCl, while animal material was more resistant. This may be a reflection of protective NH_2 groups in sugar amines in animal polysaccharides (Neuberger and Marshall, 1966). Acid hydrolysis of ^{14}C-labelled animal material yielded glucose, galactose and a pentose (similar to the products of secreted ^{14}C-mucus). These sugars were probably derived, at least in part, from mucus within the animal. Acid hydrolysis of ^{14}C-labelled plant insoluble material yielded glucose, galactose and mannose, which may have been derived in part from "cell wall" material, since *Caulerpa* is known to be rich in polysaccharides containing galactose and mannose (Percival and McDowell, 1967).

The incorporation of fixed ^{14}C into mucus is clear evidence that the animals can metabolize products released by the chloroplasts, but there is no data on how much carbon is provided by the chloroplasts for animal mucus synthesis. However, the observation that animals with chloroplasts secreted three to four times as much mucus as those without suggests that carbon supply from the chloroplasts is quantitatively important in mucus synthesis. The very rapid turnover of fixed ^{14}C in the mucus secreting pedal gland, demonstrated in the experiments, further supports this view.

The carbohydrate composition of mucus secreted by *Tridachia* and *Tridachiella* was generally similar. After hydrolysis, the sugars detected were glucose, glucosamine, galactose, galactosamine, and an unidentified pentose. Following incubations of the animals in $NaH^{14}CO_3$, photosynthetically fixed ^{14}C became chemically incorporated into secreted mucus as ^{14}C-sugars. Thus, as previously suggested by Trench *et al.*, (1970) it is possible that one of the forms in which carbon moves from the chloroplasts to the animals is as sugar or sugar precursors (see also Smith, Muscatine and Lewis, 1969). The animals very likely possess the enzyme systems necessary for their interconversions. A hypothetical scheme depicting this is shown in Figure 4. Although the mode of transfer is not known, metabolites very likely enter the haemocoel and are distributed throughout the organism. The sites at which the intercoversions may occur are also unknown, though from data available from other systems (see Hunt, 1970), it is possible that an epimerase system may be located near the cells containing the chloroplasts, while other epimerases, transaminases and dehydrogenases may be associated with the mucus secreting cells themselves. Thus, the sugar amines and sugar acids may actually be formed in the Golgi apparatus of the cells that comprise the pedal gland (see Neutra and Leblond, 1966).

There is as yet no direct measurement of the proportion of fixed ^{14}C released by symbiotic chloroplasts. However, since 30% of the total ^{14}C fixed was recovered in mucus after 5 hr in the pulse label experiment (Fig. 2), and since mucus is obviously an animal product, then a minimum of 30% of the carbon fixed by the chloroplasts in *Tridachia* is released to the animal. It should be borne in mind however, that mucus synthesis is not the only fate of chloroplast photosynthetic ^{14}C, as indicated by the radioautographic studies of Trench *et al.*, (1969). Neither is there any available data on how much of the chloroplast products is used in respiratory pathways. With the data available, it is possible to make an estimate of the proportion of fixed ^{14}C released if the following three assumptions are valid; (i) that free galactose (in the "intermediary metabolites" fraction) is an animal product made from some unknown precursor released from the chloroplasts (see Fig. 4); (ii) that galactose itself is a precursor used in the synthesis of mucus, and (iii) that the chloroplasts do not themselves synthesize large quantities of ^{14}C-polysaccharide (unpublished results of preliminary experiments on isolated chloroplasts suggest that this may in fact be the case). From these assumptions, it is possible to calculate from the data in Tables I and III that after 6 hr photosynthesis in $H^{14}CO_3^-$, 51%–54% of the total ^{14}C fixed in *Tridachia* and *Tridachiella* can be found in free galactose and mucus. It is therefore suggested that at least half of the carbon fixed photosynthetically by symbiotic chloroplasts is released to the tissues of the host animal.

The detection of free sugars and amino sugars in hydrolysates of mucus does not provide proof that these occur as the unsubstituted hexoses in native mucus. For example, N-sulphate, O-sulphate or N-acetyl groups would have been removed during acid hydrolysis. Similarly, the presence of a pentose in hydrolysates of mucus could be an indication of the occurrence of uronic acid in the native mucus, since under conditions of acid hydrolysis, uronic acids may be decarboxylated (Brimacombe and Webber, 1964).

Similar to the mucus secreted by *Placobranchus* (Trench *et al.*, 1970) mucus secreted by *Tridachia* and *Tridachiella* appears to contain a protein moiety associated with the polysaccharide polymer. The observations on the incorporation of ^{3}H-leucine into mucus-secreting tissues by *Tridachia* suggest the existence of a polysaccharide-protein complex. Most studies of mucus from molluscs have reported the presence of protein (Brimacombe and Webber, 1964; Masamune and

Yosozawa, 1965), and that from *Tridachia* and *Tridachiella* appears to conform to the pattern. Although no details of the structure of mucus from the pedal gland of *Tridachia* and *Tridachiella* can be presented, it is very likely a sulphated acid polysaccharide associated with protein.

It is important to stress that the experiments described represent studies of intact systems containing chloroplasts, and not the isolated chloroplasts themselves. To determine the fate of fixed ^{14}C in the plant and animal systems, it would be advantageous to show the form in which ^{14}C is released from the chloroplasts in animal and plant cells. Experiments designed to study these problems are now in progress.

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SUMMARY

1. The products of photosynthetic $^{14}CO_2$ fixation by symbiotic chloroplasts in the marine sacoglossan opisthobranch gastropods *Tridachia crispata* (Mörch) and *Tridachiella diomedea* (Bergh) were compared with those of the siphonous green alga *Caulerpa sertularioides*.

2. After six hours photosynthesis in $NaH^{14}CO_3$, the distribution of ^{14}C in organic compounds in the two slugs was similar, but different from that in the plant.

3. The major soluble ^{14}C-labelled carbohydrates found were glucose and sucrose in the plant and glucose and galactose in the slugs.

4. The plant contained appreciable ^{14}C-galactolipids, while little ^{14}C could be detected in galactolipids in chloroplasts in the animals. In both slugs and alga, large quantities of ^{14}C were incorporated into polysaccharide.

5. After pulse-labelleing for one hour, ^{14}C-galactose was the major soluble carbohydrate detected in *Tridachia*. Subsequently, the galactose decreased with a concomitant increase in ^{14}C-glucose. Thereafter maximum ^{14}C-glucose was detected in secreted ^{14}C-mucus suggesting utilization of galactose and glucose in mucus synthesis.

6. It is estimated that the rate of turnover of ^{14}C in the mucus-secreting pedal gland of *Tridachia* is 10–12 hours, suggesting that chloroplast products play an important role in animal mucus synthesis.

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SUPERNUMERARY LARVAL INSTARS IN CYCLORRHAPHOUS DIPTERA

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Larvae of cyclorrhaphous Diptera usually live in rapidly decaying media. They are well adapted to develop in these conditions by having a short feeding period, rapid growth rate, enormously high total metabolism, reduced number of larval instars, endomitotic growth of the larval tissues and presence of the puparium which protects the metamorphosis stages inside the sclerotized cuticle of the last larval instar. Consequently, the regulatory mechanisms for development, represented in insects by the neuroendocrine system, have undergone in this case certain modifications and deviations from the general scheme common to most other insects. For instance, in all groups of Exopterygote insects so far investigated, and also in many groups of Endopterygotes, it is possible to produce the supernumerary larval (or pupal) instars by transplanting active endocrine glands or applying juvenile hormone or ecdysone and their synthetic analogues (Pflugfelder, 1958; Novák, 1966; Sláma, 1971). However, it appears very difficult or impossible to obtain supernumerary larval instars in these cyclorrhaphous flies where such applications of the juvenile hormone resulted only in a delay of metamorphosis, or the formation of atypical puparia (Possompès, 1953), or in persistence of pupal characters in the adult (Srivastava and Gilbert, 1969; Ashburner, 1970). Ecdysone and its analogues, which have been most intensively studied and in fact isolated on the basis of the *Calliphora* assays, are merely known to cause puparium formation connected occasionally with an incomplete contraction of the puparium (Fraenkel, 1935; Butenandt and Karlson, 1954; Thomson and Horn, 1969), but no extra-larval ecdysis. Possible arguments for explaining the difficulties in producing supernumerary larval instars in this group traditionally focused on limitations of the ploidy in the endomitotic type of growth, or on the constant, and perhaps genetically fixed, number of larval instars.

Our approach to the problem of supernumerary instars in higher Diptera began with an analysis of the conditions required for obtaining similar effects in other insects. The most relevant for our study was the critical period after which both the juvenile hormone and ecdysone were unable to affect the developmental program in a given instar. The critical period for determination of the pupal or adult characters occurs soon after ecdysis, especially in rapidly developing species. It can generally be prolonged or postponed by factors like low temperature, starvation or inhibition of hormonal release (Sláma, 1971). For these reasons we have investigated conditions influencing the initiation of metamorphosis in the last larval instars of two species of flies.

MATERIAL AND METHODS

Larvae of the blowfly, *Calliphora vomitoria* (L.), and the fleshfly, *Sarcophaga argyrostoma* Robineau-Desvoidy, were bred on pork liver at 26° C. The freshly ecdysed larvae of the third instar were obtained from fully grown second instar larvae which were removed from the liver and placed on wet filter paper before ecdysis. The identification of separate larval instars was made according to the morphological criteria described by Possompés (1953). The starved larvae were kept on filter paper in Petri dishes containing a water vial plugged with cotton.

Ecdysterone (natural product isolated from *Polypodium vulgare* L. by Dr. J. Jizba) dissolved in a 10% ethanol solution was injected by means of finely drawn glass pipettes. The larvae were previously immobilized by chilling.

The material for histological examination was placed in Carnoy fixative, embedded in paraffin, and the sections were stained by Mallory's trichrome and Feulgen methods.

RESULTS

All the experiments described were performed on *Calliphora* and most were repeated on *Sarcophaga*. The results were almost identical for both the species and thus we present the quantitative data only for one of them, *i.e., Calliphora.*

1. *Effects of feeding on the initiation of development*

The blowfly larvae develop in three instars lasting approximately 18, 24, and 130 hours, respectively. They feed from hatching until the third day of the last instar, then leave the food and form puparia about 2 days later (Fig. 1).

It is generally known that feeding may stimulate the release of hormones regulating developmental cycles in larval instars and reproductive cycles in adults. At the beginning we have attempted to find out whether that was so in the case of third instar larvae of the blowfly. As is seen in the results in Figure 1, the larvae starved from the moment of last larval ecdysis survive for many days without any sign of further development. When such starved larvae are allowed to feed, they resume development leading to puparium formation. The timing of the developmental events is roughly the same as in normal third instar larvae, except that pupariation is delayed for a period corresponding to the period of starvation. Manifestly, the development leading to puparium formation is dependent on feeding after the last larval ecdysis.

In further experiments we determined the minimum amount of feeding necessary to initiate the developmental process. We found that as little as 3 to 10 hours of feeding could in certain cases lead to formation of small puparia from which emerge dwarf adults. Forms intermediate between larva and puparium were never observed. These findings suggest that the mechanisms for the metamorphosis process as a whole are irreversibly activated by a brief period of feeding after the last larval ecdysis.

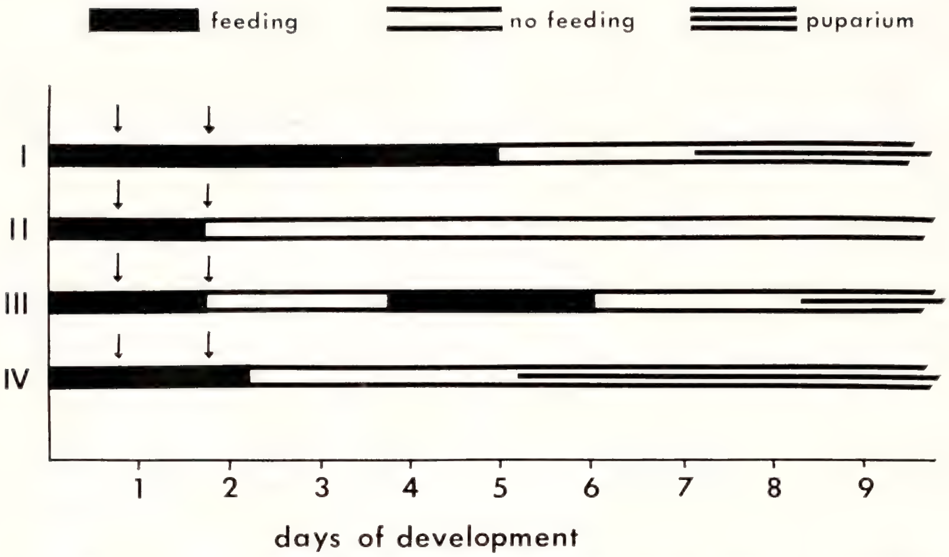

FIGURE 1. Diagrammatic illustration of the relationships between feeding and development in larvae of *Calliphora vomitoria*; I, normal development; II, non-developing larvae starved since the last larval ecdysis; III, larvae temporarily starved for determined period in the 3rd instar; IV, larvae allowed to feed only 12 hours after the last larval ecdysis. The arrows indicate larval ecdyses.

2. *Combined effects of starvation and high doses of ecdysterone*

Small amounts of ecdysone or ecdysterone are known to stimulate the metamorphosis processes. Relatively large doses cause, as a rule, precocious ecdysis with the form of the body corresponding to the degree of developmental determination at the moment of injection. Therefore, we applied large doses of ecdysterone to larvae of the third instar before the mechanisms which determine metamorphosis were activated. From our previous observations we calculated that this period must occur at the very beginning of the last larval instar or in

TABLE I

Effects of feeding on the action of high doses of ecdysterone (20 μg per larva) in the 3rd larval instar of Calliphora

Feeding period and ecdysterone injection (hr after ecdysis)	No. of larvae	Effects 24 hours after injection			
		Supernumerary larvae	Intermediates	Puparia	Dead
0	17	12	0	0	5
3	21	15	1	0	5
6	27	16	8	0	4
12	26	0	14	4*	7
24	25	0	0	22	2

* Incompletely contracted.

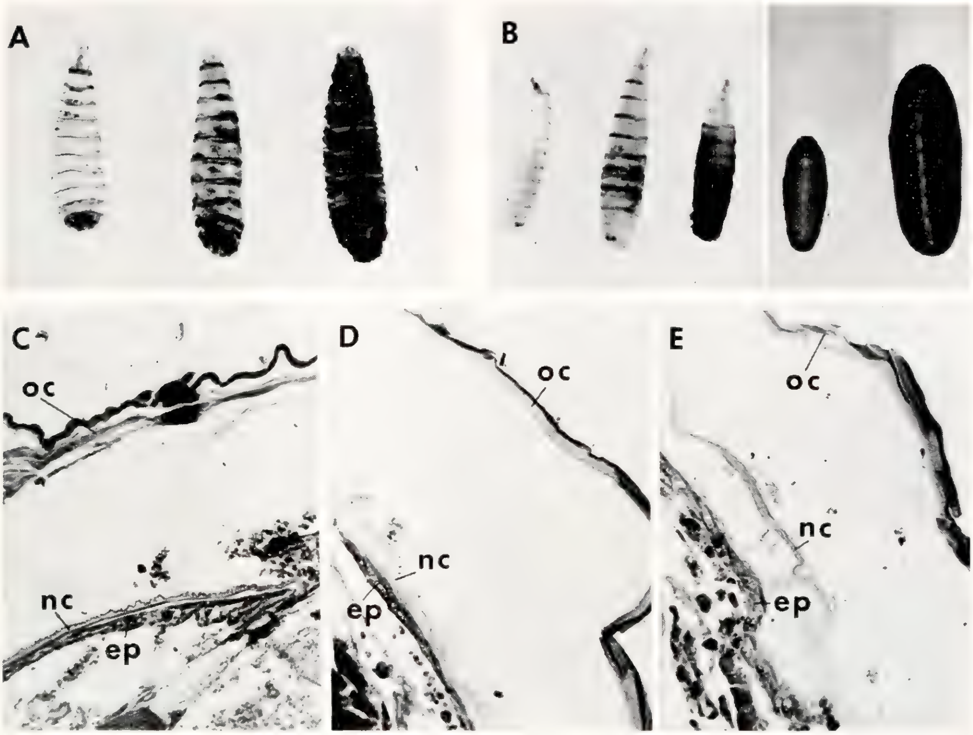

FIGURE 2. Supernumerary larvae and larvae-puparium intermediates in *Sarcophaga* and *Calliphora;* (A) larva-puparium intermediates of *Sarcophaga* with different degree of pupariation; (B) supernumerary larva, two larva-puparium intermediates, dwarf puparium and normal puparium in *Calliphora*; (C) section through integument of *Sarcophaga* larvae after ecdysterone injections showing the old 3rd instar cuticle and the new extra-larval cuticle; (D) the same in *Calliphora*; (E) similar section in *Calliphora* showing the detachment of the extra-larval cuticle from the epidermal cells. Abbreviations are: oc—old cuticle of the 3rd instar larva, nc—new extra-larval cuticle, ep—epidermal cells.

starved last instar larvae. As a matter of fact, injections performed at these stages induced precocious molt and secretion of an extra-larval cuticle (Table I). The supernumerary larvae produced in this way were of the same size as newly ecdysed third instar larvae. The new cuticle was apparent approximately 24 hours after the injection, but the larvae were unable to shed the non-sclerotized and incompletely digested old larval cuticle. The newly formed extra-larval cuticle had perfect larval characteristics such as a set of stigmatic plates on the hind spiracles, tranverse bands of segmental spinules, *etc.* (Fig. 2).

Similar injections of ecdysterone into larvae which received a subcritical amount of feeding (3 hours) also produced supernumerary larval instars. By increasing the time of feeding we obtained supernumerary larvae and intermediates with a wide range of mosaic pupariated spots in the old cuticle. With even more feeding we obtained intermediates possessing more of the pupariated spots in the old cuticle and no supernumerary larvae. And, finally, after 24 hours of feeding more or less normal puparia were formed (Table I). These results

TABLE II

Effects of high and low doses of ecdysterone on starved (non-developing) 3rd instar larvae of Calliphora

Ecdysterone (in μg per larva) injected at		Effects observed 24 hours after the last injection		
0 hours after ecdysis	72 hours after ecdysis	Formation of new cuticle	Developmental stage achieved	Degree of pupariation*
20	0	Yes	Supernumerary larva	0%
0	20	Yes	Supernumerary larva	0%
5	0	No	Permanent larva	
0	5	No	Permanent larva	
1	20	Yes	Larva-puparium intermediate	10%
2	20	Yes	Larva-puparium intermediate	30%
5	20	Yes	Larva-puparium intermediate	20%
1	2	Yes	Larva-puparium intermediate	30%
2	2	Yes	Larva-puparium intermediate	50%
5	2	Yes	Larva-puparium intermediate	70%

* Based on the estimated tanned area of the old cuticle.

with high doses of ecdysterone demonstrate that when the larvae are able to feed, the initiation of processes of metamorphosis and the associated loss of ability of the epidermal cells to form the larval patterns are taking place in the 24 hour interval after the last larval ecdysis.

In further experiments we tried to find out to what extent was the decision-making mechanism which would either maintain the *status quo* or commit the cells and tissues to metamorphosis dependent on feeding. In these experiments larvae starved from the moment of ecdysis were injected with high doses of ecdysterone after 24 or 72 hours of starvation. In all cases we got only perfect supernumerary larvae (Table II). Evidently, the whole endogenous mechanism for the determination of metamorphosis remains inhibited in the absence of food.

The individuals destined to form supernumerary larvae or intermediates underwent a precocious molt characterized by apolysis, more or less profound digestion of the old cuticle, and the formation of a thin new cuticle (Fig. 2 C, D). All the larvae treated with high doses of ecdysterone were unable to feed; they had limited ability to move, and were unable to shed the old cuticle. They died after 2 to 3 days. When they were dissected we got an impression that the supernumerary larva showed apolysis of its cuticle. The latter in some cases could be removed and underneath remained a delicate integumentary layer. This was confirmed on some histological sections. Thus, as seen in Figure 2 E, the extra-larval cuticle is detached from the epidermal cells and partially digested. In the case of larval-puparium intermediates the old partially pupariated cuticle was always separated from the epidermis. The newly formed cuticle was never pupariated.

3. *Effects of small doses of ecdysterone*

After we realized that the endogenous determination process was suppressed in starved larvae, we examined the effects of small doses of ecdysterone. While doses of 20 μg per larva always induced precocious ecdysis, doses of 5 μg and less did not have this effect.

The data in Table II demonstrate that small doses of ecdysterone injected after ecdysis, combined with large doses injected 3 days later, produced intermediates with various degrees of tanning in the old cuticle. It is interesting to note that as little as 1 μg of ecdysterone injected after ecdysis affects the metamorphosis determination process in certain areas of epidermis; it results ultimately in tanning of the old cuticle above the affected epidermal cells. Combinations of two small doses, one after ecdysis and the other 3 days later also led to the larva-puparium intermediates, although the total amount of ecdysterone injected was far below the amount necessary for precocious ecdysis. As seen at the bottom of Table II, the greater the dose of ecdysterone injected immediately after ecdysis, the more extensive was the area of tanned cuticle of the puparial type. Thus, by administering repeated low doses of ecdysterone to these starved larvae, we succeeded in partially imitating the endogenous mechanism regulating the initial processes of metamorphosis in these flies.

DISCUSSION

Our results indicate that the number of larval instars in cyclorrhaphous flies is under the control of the neuroendocrine system. The hormonal mechanism synchronizing development with the availability of food has been known for a long time in diverse insect species (Thomsen, 1952; Johansson, 1958; Strangways-Dixon, 1961; Engelmann, 1968).

We have found that the "gene-set switching mechanism" for metamorphosis occurs in these flies very early after the last larval ecdysis when enough food is available. The perfect supernumerary larvae can be produced only by excessive amounts of ecdysterone and only when this is administered just after the ecdysis or injected into starved third instar larvae. With feeding third instar larvae older than 24 hours it was impossible to obtain anything but puparia. In addition, small amounts of ecdysterone never caused perfect supernumerary larvae, but in combination with repeated administration of ecdysterone the larvae formed more or less advanced larva-puparium intermediates. This confirms the qualitative difference between the effects of excessive and small amounts of ecdysones (Williams, 1968). We conclude that the excessive amounts of ecdysterone cause immediate realization of the existing genetical information. By contrast, the small amounts of the hormone cause progressive switching on of the gene-sets responsible for the metamorphosis development.

During the normal development the third instar larvae most likely maintain a low level of endogenous ecdysones conditioning the programming of processes leading to puparium formation. In some instances, the hormone may temporarily disappear and the puparium formation is consequently delayed, as is the case of *Sarcophaga* larvae kept in contact with water (Ohtaki, Milkman and Williams, 1968; Zdarek and Fraenkel, 1970) or diapausing larvae of *Lucilia* (Fraser and Smith, 1963). The fact that so many investigators applying ecdysones to final larval instars of higher Diptera failed to obtain the supernumerary larval instars may be explained in the following way. Most investigators used advanced third instars where the developmental program for metamorphosis had already been determined. Secondly, they may not have expected that the required amounts of the hormone would be so extremely high. To get a positive result in the puparium assay on *Calliphora* larvae, one needs to inject approximately 0.01 μg of ecdysterone per ligated abdomen. However, in order to get supernumerary ecdysis in starved larvae of *Calliphora* (which is about 10 times smaller in weight) we need at least 10 μg of the hormone, *i.e.*, 10,000 times more per unit of weight.

We are greatly indebted to Prof. C. M. Williams of Harvard University for helpful criticism and corrections of the English.

SUMMARY

1. The developmental program for metamorphosis is determined in third instar larvae of *Calliphora* and *Sarcophaga* very early after ecdysis. The process is activated by feeding and can be delayed for many days by starvation.

2. High doses of ecdysterone (20 μg per larva) injected just after ecdysis or in starved larvae cause a precocious molt and the formation of supernumerary larvae. The same treatment of larvae a few hours after ecdysis leads to larva-puparium intermediates displaying the mosaic of tanned areas in the old cuticle. Larvae older than 24 hours after ecdysis responded only by forming small puparia.

3. Small doses of ecdysterone (less than 5 μg per larva) do not cause a complete molt but activate the "gene-set switching mechanism" for programming of the metamorphosis development. The degree of such a covert effect can be revealed by repeated injections of ecdysterone which lead to formation of more or less advanced larva-puparium intermediates.

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HEPATOPANCREAS OF *PARATELPHUSA HYDRODROMOUS* (HERBST): HISTOPHYSIOLOGY AND THE PATTERN OF PROTEINS IN RELATION TO REPRODUCTION AND MOLT

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A major seat of bulk storage, syntheses and transformations of a variety of organic and inorganic substances, the hepatopancreas of crustaceans, which is analogous to the liver of vertebrates and the fat body of insects, may aptly be described as a metabolic factory *par excellence* (Lockwood, 1967; Huggins and Munday, 1968; Adiyodi and Adiyodi, 1970). Hepatopancreatic reserves, which are maximal in intermolt (stage C_4) are mobilized to meet the demands of somatic and reproductive growths, and also exigencies like inanition and perhaps also altered environmental conditions. The free sugars (Adiyodi and Adiyodi, 1970a) and phospholipids (Adiyodi and Adiyodi, 1970b) in the hepatopancreas of the crab, *Paratelphusa* show variations, both qualitative and quantitative, related to vitellogenesis, suggesting that these substances may be utilized in some way in the formation of yolk. Cyclic fluctuations occur in the levels of organic (Adiyodi, 1969a) and inorganic (Adiyodi, 1969b) reserves in the hepatopancreas of *Paratelphusa* associated with the periodic molt. Our earlier studies have shown that the major organic resources in the hepatopancreas of *Paratelphusa* are lipid (Adiyodi, 1969a) and free sugars (Adiyodi and Adiyodi, 1970a). Of the constituents analyzed, the neutral fats (Adiyodi, 1969a) and phospholipids (Adiyodi and Adiyodi, 1970b) predominate among the lipid, the free unsaturated fatty acids and cholesterol occurring in medium and low levels, respectively (Adiyodi and Adiyodi, 1972).

In insects a dynamic relationship exists between the hemolymph and the fat body proteins (Chippendale and Kilby, 1969). Changes have been described in the profiles and proportions of proteins in the plasma of *Paratelphusa* (see Adiyodi and Adiyodi, 1970, for references) and several other species of decapod Crustacea, related to molt and reproduction (Barlow and Ridgeway, 1969; Kerr, 1969). The hepatopancreas has been implicated in the synthesis of proteins in Decapoda (Kurup and Scheer, 1966). But the nature of the relationship existing between proteins in the hepatopancreas and the blood has been little investigated. Our

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knowledge on the histology, ultrastructure and storage function of the hepatopancreas is also meagre in the Decapoda, except perhaps in the crayfish, *Procambarus clarkii* (Ogura, 1959; Miyawaki, Matsuzaki and Sasaki, 1961; Miyawaki and Tanoue, 1962). This study has as its main objectives the exploration of the nature and extent of protein synthesis and "storage" in the hepatopancreas, interrelationship between these proteins and those of the plasma, their utilization in molt and reproduction, their fate during starvation, and the influence of eyestalk hormone or hormones on their accumulation and utilization. The overall role of the hepatopancreas of *Paratelphusa* as a synthesis-depository-supply center of the major organic resources is also discussed with special reference to the demands of somatic and reproductive growths.

MATERIALS AND METHODS

Material

The crabs used were mostly fresh from paddy fields, and included juveniles and adults of both sexes. The stage in the molt cycle was determined according to Drach's (1939) schedule. Yolk deposition (which has been used as a parameter in our studies) may be divided into two phases, Vitellogenesis I (subdivided into stages 1–3) and Vitellogenesis II. Stage 1 oocytes are white in color; stage 2 oocytes are a pale yellow, and show the beginnings of protein yolk synthesis. Stage 3 oocytes acquire an orange color and reach an average diameter of 1 mm. Vitellogenesis II, which follows stage 3 is a period of rapid yolk deposition, terminating eventually in oviposition.

Histochemical and biochemical procedures

The hepatopancreatic lobules of crabs in different stages of the molt cycle were fixed in 10% neutral formalin or chilled absolute alcohol. The dehydration of tissues fixed in the latter was carried out at temperatures ranging from 3–5° C. Paraffin sections, 5 μ in thickness, were stained with Millon's reagent (Baker modification), mercury-bromophenol blue (after Bonhag) (MBB), Sudan black B (after McManus) (SBB), and periodic acid-Schiff (Lillie's method) (PAS) (Pearse, 1968). Millon's, xanthoproteic and biuret tests were employed for biochemical detection of proteins in water homogenates of hepatopancreatic tissues (Oser, 1965).

Electrophoresis

Soluble proteins of the hepatopancreas, ovary and blood of crabs in different stages of molt cycle and reproductive activity were analyzed qualitatively by disc electrophoresis on polyacrylamide gels. The ovary and hepatopancreas were removed from crabs, which had been prechilled for 10 minutes, into cold Ringer. They were washed well with glass distilled water and homogenized in an all glass homogenizer. The homogenates were centrifuged in the cold (3–5° C) at 5000 rev/minute for 20 minutes. Eight μl of the supernatant was used for the fractionations.

Electrophoresis was conducted in a Canalco Model 6 Disc Electrophoretic Unit for 20 minutes under a constant current of 6 mamp/column using β alanine acetate as the buffer (pH 4.5). In the case of the hemolymph, the cellular elements were spun off by centrifugation in the cold (3–5° C), and only the clear plasma (8 μl) used. After electrophoresis the gels were immersed in a 0.1% solution of amido black (AB) in 7.5% acetic acid (2 hours). Background stain was removed electrophoretically (10 mamp/gel). For the detection of lipoproteins the gels were immersed in a saturated filtered solution of SBB in 70% ethanol for 24 hours, destained in 70% ethanol, and transferred to 7.5% acetic acid for hydration and storage. For glycoproteins, the gels were first placed for 1 hour in 7.5% acetic acid soon after electrophoresis, and for another hour in 0.2% periodic acid (4° C); after a short rinse in 7½% acetic acid they were transferred to Schiff's reagent (4° C) for 12 hours (PAS). Fractions have been numbered serially from the origin, based on their relative mobilities (R_{mb}) in th electric field. In the nomenclature employed here, H, P and OV denote proteins of the hepatopancreas, plasma and ovary, respectively.

Eyestalk removal

Eyestalks were removed from juvenile crabs in stage C_4 and a few adults, one eyestalk at a time, with an interval of 24 hours between the two operations on each individual, with a view to study the influence of eyestalk hormones on protein synthesis and utilization. This method of operation reduced the postoperative mortality to about 5%. The experimentals and controls (normal crabs) were maintained on a diet of earthworms (*Megascolex* sp.).

Starvation

Seven adult female crabs in their intermolt were individually reared in glass jars for 3 months on a bed of moist filtered sand. The crabs had ready access to water, but no food was provided. At the beginning of the experiment, the stage of the ovary was ascertained by the window method (Gomez and Nayar, 1965).

RESULTS

Histology and histochemistry of the hepatopancreas

In normal well-fed adult female crabs nearly three-fourths of the body cavity is filled by the hepatopancreas. Usually it has an orange hue, but its color may change with the stages in the molt cycle. Hepatopancreas is deep brown or black in postmolt, orange in intermolt (stage C_4) and black in late premolt. Such color differences could hardly be detected in starved or ill fed crabs, the hepatopancreas of which appeared a dull pale yellow. The hepatopancreas of some of the destalked crabs became whitish on molt, despite the fact that the animals were well cared for during the postoperative period. Possibly, this is related to the fact that the crabs become hypophagic after the initial hyperphagia on eyestalk removal, and the hepatopancreatic reserves become consequently much depleted.

Longitudinal sections of the hepatopancreatic lobules of *Paratelphusa* show the presence of three separate zones, as already briefly reported (Adiyodi, 1969a). Zone I is composed of diminutive, closely packed embryonic cells, and Zone II largely of secretory cells or Blastenzellen. In Zone III, which constitutes the major portion of the lobule, two types of cells corresponding to the metal cells

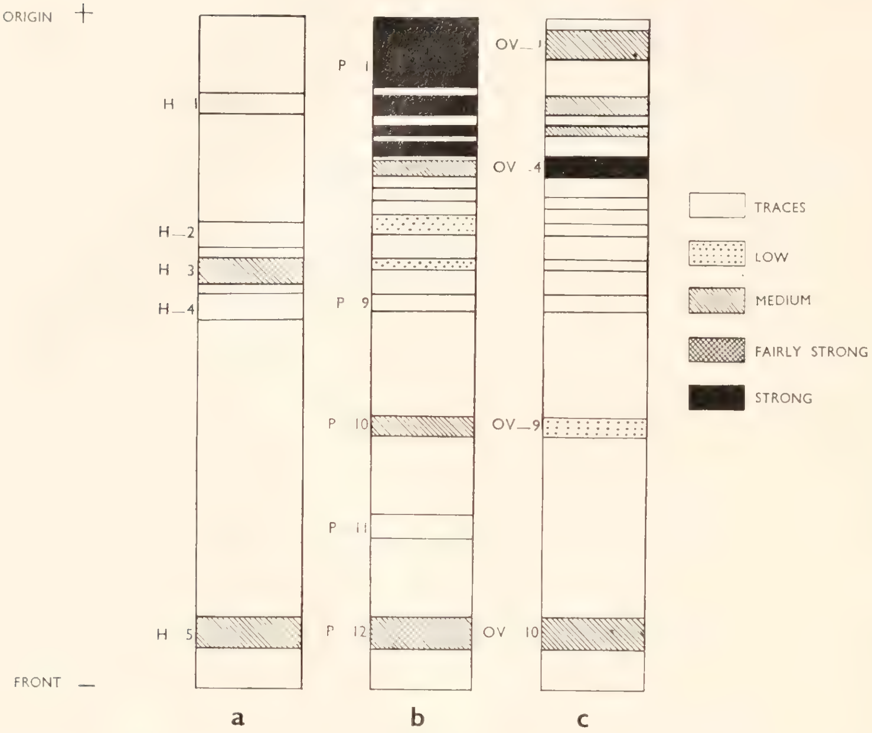

FIGURE 1. Electropherograms of *Paratelphusa hydrodromous* showing the pattern and homology of proteins resolved at pH 4.5 in different tissues; (a) hepatopancreas (diagrammatic showing all the fractions); (b) plasma (in stage 3 of Vitellogenesis I) and (c) ovary (in stage 2 of Vitellogenesis I).

(iron cells and copper cells) described in the midgut gland of the crayfish, *Procambarus clarkii* by Ogura (1959) and also Miyawaki, Matsuzaki and Sasaki (1961) are observed. The iron cells of *Paratelphusa* have a nucleus roughly 7μ in diameter and a large nucleolus; the cytoplasm is rather homogeneous and strongly basophilic. Copper cells have smaller nuclei (about 4μ in diameter) and vacuolated cytoplasm, the vacuoles often being filled with stainable substances. The metal cells in Zone III serve as storage centers of both organic and inorganic (*e.g.,* calcium) reserves.

During stage C_4 the interior of the metal cells is literally filled with substances positive to SBB and PAS. With MBB and Millon's, on the other hand, the general stainability of the metal cells was very low even in stage C_4. Distinct droplets strongly positive to MBB and Millon's were observed in small amounts in the interlobular spaces, but very rarely inside the metal cells. These droplets were positive also to SBB and PAS. The observations reported here indicate a low level of stainable protein in the hepatopancreatic tissue. This was further substantiated by the biochemical tests for proteins (Millon's, xanthoproteic and biuret reactions) on whole tissue homogenates.

Soluble proteins of the hepatopancreas in relation to reproduction

Electropherograms of the hepatopancreas showed a total of five protein fractions (H-1 to H-5) (Fig. 1a). The plasma samples of female crabs with ovaries in stage 3 run simultaneously under the same system for reference and comparison showed 12 fractions (P-1 to P-12) (Fig. 1b). Stage 2 ovaries showed the maximum number of protein fractions (10) (OV-1 to OV-10) (Fig. 1c). Fractions OV-1 to OV-3 appear to be homologous in their R_{mb}s to P-1 to P-3, respectively, OV-4 to both P-4 and P-5, OV-5 to OV-9, respectively, to P-6 to P-10, and OV-10 to P-12. Fractions H-1 to H-5 of the hepatopancreas were found to correspond in their R_{mb}s to fractions P-2, P-7, P-8, P-9 and P-12 of the plasma, respectively, and to fractions OV-2, OV-6, OV-7, OV-8 and OV-10 of the ovary, respectively. The fastest moving fraction H-5 resolved in some gels as a heterogeneous chromoprotein. Reaction with SBB and PAS was negative at all stages of the reproductive cycle suggesting that fractions H-1 to H-4 are only simple proteins.

Homogenates of the hepatopancreas of juvenile females (Fig. 2a) and adult males (Fig. 2b) yielded during the intermolt generally two AB-stainable fractions, H-3 and H-5, the former only in extremely low levels. The hepatopancreas of juvenile males (Fig. 3c) in their intermolt yielded sometimes besides these fractions also fraction H-2, though in traces. The pattern of soluble proteins in

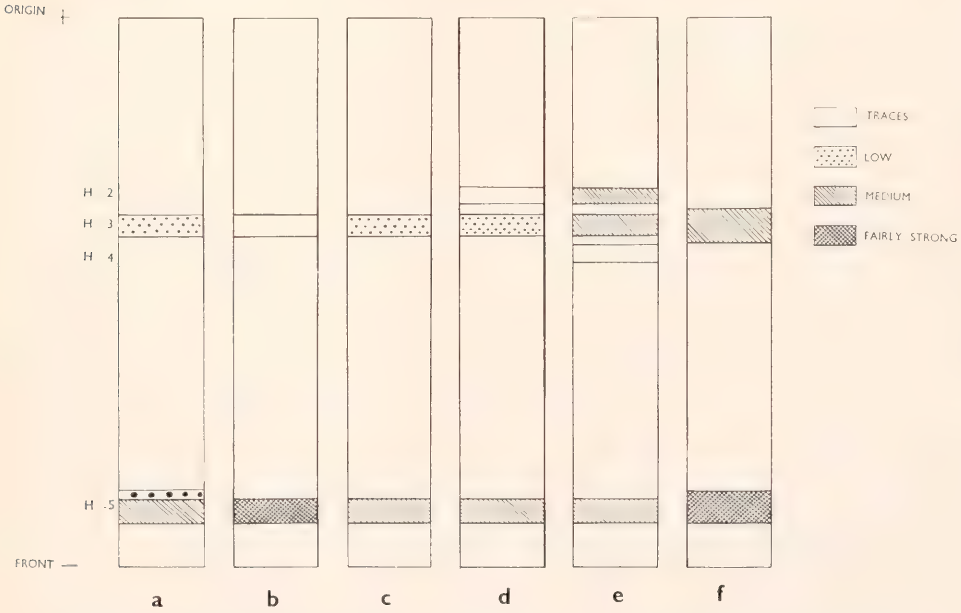

FIGURE 2. Electropherograms of the hepatopancreas of *Paratelphusa hydrodromous;* (a) juvenile females in stage C_4; (b) adult males in stage C_4; (c) adult females with the ovaries in stage 1; (d) adult females with the ovaries in stage 2; (e) adult females with the ovaries in early stage 3 and (f) adult females with the ovaries in mid and late stage 3 of Vitellogenesis I.

the hepatopancreas of intermolt adult females with their ovaries in stage 1 (Fig. 2c) was comparable to that of juvenile females and adult males. Fraction H-2 appeared with stage 2 (Fig. 2d). With the ovaries in early stage 3 (Fig. 2e) four fractions could be detected in the hepatopancreas (H-2 to H-5); but in mid-stage 3 individual fractions, H-2 and H-4, were hardly distinguishable (Fig. 2f). Mid and late stage 3 crabs had more AB-stainable proteins in fractions H-3 and H-5 than forms in early stage 3.

Soluble proteins of the hepatopancreas in relation to the molt schedule

Only juvenile crabs were used for these studies. The hepatopancreatic electropherograms of early (stage A and B) (Fig. 3a) and late postmolt (stages C_1–C_3) (Fig. 3b) crabs were remarkably similar to those obtained in intermolt (stage C_4) (Fig. 2a). In juvenile females the biggest peak was represented during stage C_4 by the fast moving fraction (H-5); fraction H-3, the other component present, was in traces. There occurs a decrease in the concentration of fraction H-3 during early premolt (D_0–D_1) (Fig. 3d). As the crabs entered D_2–D_3 this fraction registered a sharp increase (Fig. 3e). Late premolt (stage D_4) was characterized by the appearance of a slow-moving high molecular protein (fraction H-1), not hitherto represented in the electropherograms of the hepatopancreas (Fig. 3f). In some crabs this was attended with a slight fall in the levels of fraction H-5.

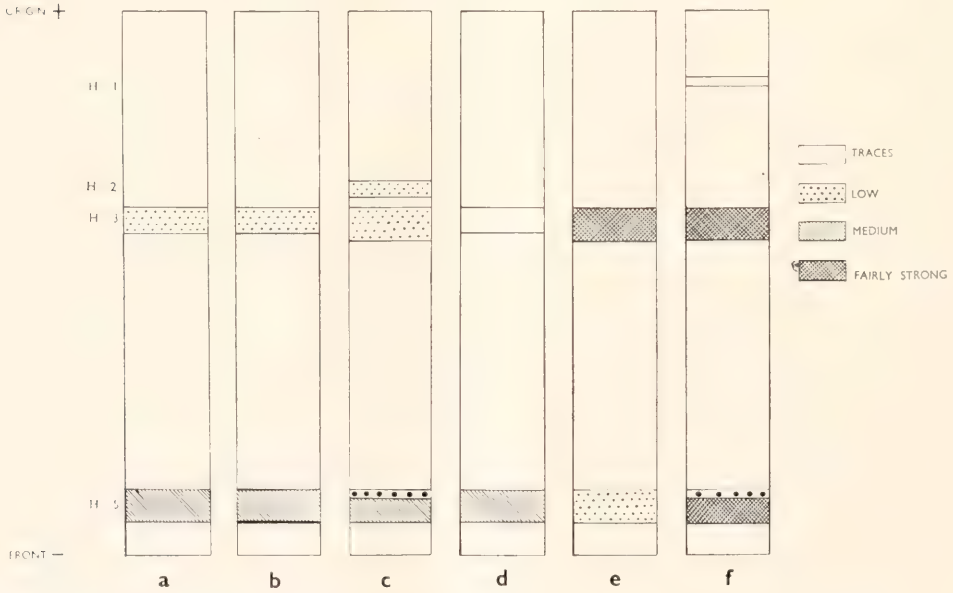

FIGURE 3. Electropherograms of the hepatopancreas of *Paratelphusa hydrodromous* in relation to the molt schedule; (a) juvenile females in stage A–B; (b) juvenile females in stage C_1–C_3; (c) juvenile male in stage C_4; (d) juvenile females in stage D_0–D_1; (e) juvenile females in stage D_2–D_3 and (f) juvenile females in stage D_4.

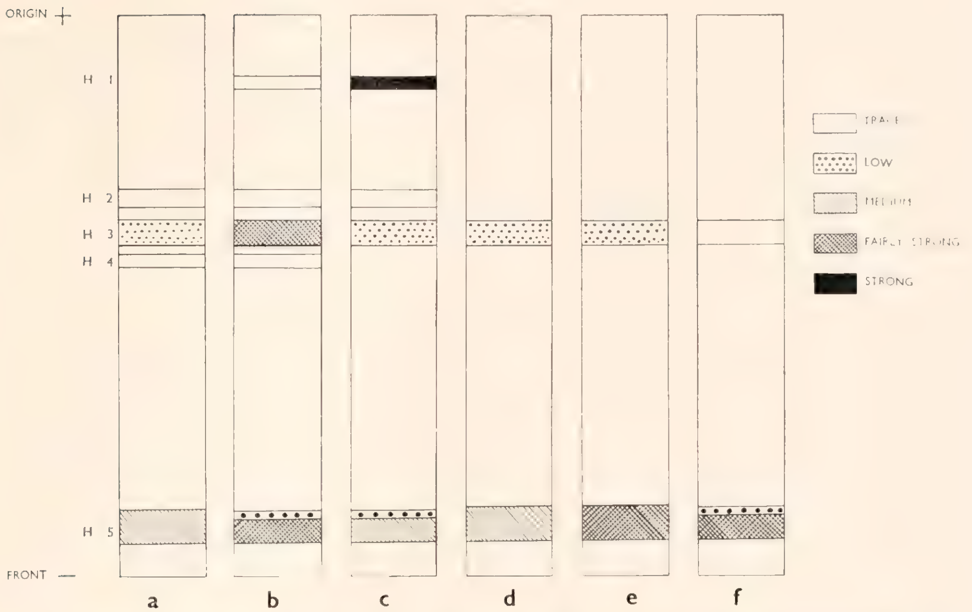

FIGURE 4. Electropherograms of the hepatopancreas of *Paratelphusa hydrodromous* under eyestalk ablation; (a) juvenile female D_0–D_1, 8 days after eyestalk removal; (b) juvenile female in D_3–D_4, 15 days after eyestalk ablation; (c) adult female, 8 days after eyestalk removal; (d) juvenile female (control) after 15 days; (e) adult male, 15 days after eyestalk ablation and (f) destalked adult female administered eyestalk extract, 3 days after the operation and assayed 5 days thereafter.

Pattern of proteins in the hepatopancreas of destalked crabs

Homogenates of the hepatopancreas of 7 juvenile female crabs were analyzed electrophoretically, 3 on the 8th day and the remaining 4 on the 15th day of ablation of both the eyestalks. Some of the electropherograms showed all the 5 fractions (H-1 to H-5), fraction H-1 being present particularly in forms that had precipitously entered D_3–D_4 (Fig. 4b). The crabs in D_0–D_1 showed only fractions H-2 to H-5 (Fig. 4a). Four adult female crabs with ovaries in stage 1 were deprived of their eyestalks, and their hepatopancreas assayed for proteins 8 days after the operation (Fig. 4c). They were in stage C_4 of the molt cycle at autopsy, but showed a larger number of fractions than the 3 control adult female crabs, and also an accelerated entry into late stage 2 or early stage 3 of vitellogenesis. The pattern in destalked adult females simulated in some respects that of crabs with ovaries in early stage 3, but more so was the comparison with the picture obtaining in premolt juvenile crabs in that fraction H-1 was also present. None of the 5 control juvenile females (with intact eyestalks) entered premolt, the only fractions in their hepatopancreas being H-3 and H-5 (Fig. 4d). The electropherograms of the hepatopancreas of two adult males (Fig. 4e), 15 days after eyestalk removal showed, like those of their 2 controls, only two fractions, H-3 and H-5, these destalked male crabs were still in stage C_4.

It was necessary to ascertain whether the effects observed by eyestalk displacement on hepatopancreatic proteins could be reversed by substitution therapy. For this purpose an extract of eyestalks of intermolt adult females was prepared in Ringer and injected into 3 females having stage 1 ovaries, at the rate of two eyestalks in 0.01 ml Ringer/crab, 3 days after their eyestalks had been removed. The hepatopancreas was assayed 5 days after the administration of the extract. The hepatopancreatic electropherograms of the destalked females which received the eyestalk extract (Fig. 4f) were almost indistinguishably like those of normal intermolt adult females with stage 1 ovaries (Fig. 2c) in that there were only two fractions, H-3 and H-5.

Effect of starvation on hepatopancreatic soluble proteins

All the crabs subjected to inanition had their ovaries at the beginning of the experiment at stage 3 (early, mid and late) of vitellogenesis I. Prolonged food withdrawal led not only to an arrest in oocyte growth, but also to yolk lysis and oosorption in as many as 5 out of the 7 crabs. The hepatopancreas of the starved individuals wore a bleak paper-white appearance and showed much reduction in size and weight. Electropherograms of the hepatopancreas of animals with resorbing ovaries showed mostly only fraction H-5 (fraction H-3 being often too low to be detected), whereas in the two crabs with no visible signs of resorption, fraction H-3 was present in medium levels.

Discussion

It is well known that large amounts of organic resources accumulate in the hepatopancreas of crustaceans during the intermolt, and that such stores are mobilized to meet the demands of events like molt and reproduction (Adiyodi and Adiyodi, 1970). Though estimates of total protein content are not available, our histochemical and biochemical studies sufficiently indicate that the protein level in the hepatopancreas of *Paratelphusa* is rather low even in the intermolt. The hepatopancreas of *Paratelphusa* is rich in monosaccharides (glucose, galactose) and the disaccharide, sucrose (Adiyodi and Adiyodi, 1970a). The intensity of color developed with PAS, it may be recalled, is dependent primarily on the amount of reactive glycol structure present, the concerned reactive groups being those of the hexose sugars, glucose, galactose, mannose and the methylpentose sugar, fucose (Pearse, 1968). The hepatopancreas of *Paratelphusa* is also rich in phospholipids especially phosphatidyl choline, phospatidyl ethanolamine and lysophosphatidyl ethanolamine (Adiyodi and Adiyodi, 1970b). The hepatopancreatic lipid of this crab is semiliquid on extraction suggesting the presence of fairly high quantities of unsaturated fatty acids; notable among the nonesterified fatty acids being oleic/palmitoleic acid (Adiyodi and Adiyodi, 1972). The unsaturated fatty acids and the first two phospholipids also react positively with PAS (Pearse, 1968). The abundantly positive color reaction shown by the metal cells of *Paratelphusa* to PAS may possibly be, therefore, attributed to sugars, phospholipids and unsaturated fatty acids rather than glycogen, which was very low to be detected with Best's carmine reaction or iodine test (Adiyodi and Adiyodi, 1970a). It is not known whether the PAS-positive substances observed in our histochemical preparations included also some glycoprotein not resolved with the acidic buffer system we have used.

A comparison of the patterns of soluble proteins in the hepatopancreas, plasma and ovary (Fig. 1) and their changes in relation to the reproductive (Fig. 2) and molt (Fig. 3) cycles (Adiyodi and Adiyodi, 1970, for references) permits some conclusions. Fractions corresponding to the vitellogenins (P-1 to P-5 or OV-1 to OV-4) were not represented in the hepatopancreas, except for the relatively minor fraction H-1, which is homologous to P-2 of the plasma and OV-2 of the ovary. Further, fraction H-1 is an unconjugated protein, whereas in the plasma its homologue, P-2 is in the form of a glycoprotein in stage 2, and in late periods of vitellogenesis; in the ovary OV-2 assumes the characteristics of a glycoprotein in late stage 3 and in Vitellogenesis II (Adiyodi, 1968a). The possibility that fraction H-1 may contribute to the plasma fraction P-2 in *Paratelphusa* can hardly be discounted. But our studies clearly suggest that the plasma sex protein (P-4, 5) which contributes significantly to yolk formation as also the other vitellogenins resolvable under acidic conditions of electrophoresis, are neither synthesized nor stored as such in the hepatopancreas. In this respect, the hepatopancreas of *Paratelphusa* differs from the vertebrate liver, which is the principal organ synthesizing serum albumin (Milhaud, 1964) and the insect fat body (Chippendale and Kilby, 1969), which synthesizes and supplies proteins to the ovary.

The increase in the levels of fraction H-3 with vitellogenesis, and the fact that fractions H-2 and H-4 make their appearance in adult females only in relation to specific stages in the ovarian cycle, suggest that these proteins may play some role in vitellogenesis, either as enzymes catalyzing some of the biological conversions, as carrier proteins in the plasma, or as fuel. The sudden decrease in the concentration of hepatopancreatic protein fractions H-3 and H-5 with the onset of premolt may perhaps be due to their increased utilization during this period of the formation of the new epicuticle. The appearance of fraction H-1 and the increase in the levels of soluble proteins in stage D_4 may be associated, at least in part, with the withdrawal of protein from the resorbing old cuticle into the hepatopancreas by way of the hemolymph. The appearance of additional plasma protein fractions or increased plasma protein levels associated with molt is already documented in insects (Siakotos, 1960) and crustaceans (Barlow and Ridgeway, 1969). In the land crab, *Gecarcinus lateralis*, according to Skinner (1965) the incorporation of valine-1-C^{14} and leucine-1-C^{14} into the hepatopancreatic proteins reaches its maximum by D_0–D_2. It is not known whether the proteins accumulated in the hepatopancreas of *Paratelphusa* in premolt include any enzymes related to molt or digestion. But the fact that in the molt cycle the protein fraction H-1 normally appears only associated with premolt points to a possible influence of the molt hormone on its accumulation.

The precocious appearance of additional fractions in intermolt juvenile and adult females consequent on eyestalk removal (which in adult females has been demonstrated to be readily reversible by substitution therapy) suggests that the synthesis of proteins in the hepatopancreas related to molt and reproduction are normally inhibited during the intermolt by some principles in the eyestalk, possibly the molt inhibiting and gonad inhibiting hormones. Such a view is further supported by the observation of McWhinnie and Mohrherr (1970) in the crayfish, *Orconectes virilis* that the eyestalk extract of intermolt animals reduces amino acid incorporation into the hepatopancreas of premolt crayfish. The fall observed in the levels of hepatopancreatic lipids (R. G. Adiyodi, unpublished) and sugars (Adiyodi and

Adiyodi, 1970a) in destalked *Paratelphusa* suggest that these substances are more readily pressed into service as fuels to meet the metabolic demands arising from eyestalk removal.

Starvation is known to reduce the rate of metabolism in Crustacea (Vernberg, 1959). In *Hemigrapsus* continued starvation for 23 days exercised little effect on glycogen content or lipid reserves, but in females there was a depletion of proteins (Neiland and Scheer, 1954). In *Paratelphusa* inanition for 3 months results in a fall in the lipid content of the hepatopancreas and a rise in PAS-positive substances, the latter possibly due to lipid breakdown (Adiyodi, 1968b). Though the observations reported here give no clue to the sequence in which the hepatopancreatic metabolites are utilized, they nevertheless indicate that the synthesis and/or accumulation of both lipid and protein become impaired. Possibly, the depletion observed in the hepatopancreatic protein with starvation may be related to its breakdown and drainage into other tissues including the hemolymph to counteract the stress on osmotic equilbrium.

We thank Prof. K. J. Joseph for his interest in the work.

SUMMARY

1. Protein level in the hepatopancreas of *Paratelphusa* is low even in intermolt. A total of 5 protein fractions (H-1 to H-5) could be detected under acidic conditions of disc electrophoresis. They are homologous to P-2, P-7, P-8, P-9 and P-12 of the plasma, and OV-2, OV-6, OV-7, OV-8 and OV-10 of the ovary, respectively, in their mobilities. None of the major vitellogenins including the plasma sex protein, which contributes significantly to yolk formation, is either synthesized or stored as such in the hepatopancreas. The increase in the levels of fraction H-3 with vitellogenesis, and the fact that fractions H-2 and H-4 appear in adult females only in relation to specific stages in the ovarian cycle, however, suggest that these proteins may play some role in vitellogenesis, as enzymes, carrier proteins or fuels.

2. Eyestalk removal causes in juvenile and adult females of *Paratelphusa* the addition of fractions H-1, H-2 and also H-4 to fractions H-3 and H-5 already present. The synthesis of the proteins in the hepatopancreas related to molt and reproduction appears to be normally inhibited during the intermolt by some eyestalk principle.

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ON THE NEUROSECRETORY SYSTEM OF *RIVULOGAMMARUS SYRIACUS* CHEVREUX

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Studies on neurosecretion in Crustacea were initiated by Perkins (1928) and Koller (1928). Later, Hanstrom's discovery (1931) of the eyestalk hormone in the sinus gland of brachyurans encouraged Brown (1944); Passano (1951); Bliss and Welsh (1952) and others to establish a neurosecretory sinus gland system in crustacea similar to the intercerebralis-cardiacum-allatum system of the insects and to the hypothalamic-hypophyseal system of the vertebrates. Since then, several morphological studies have been made on the neurosecretory system of decapod crustaceans. The significance of the role of the neurosecretory activities in the physiology of these animals has already been emphasized by several workers (Scharrer, 1955; Welsh, 1955; Knowles and Carlisle, 1956).

In Isopoda, study of protocephalic neurosecretory pathway and the corresponding endocrine glands revealed a pleomorphism greater than that observed in any other malacostracan crustaceans (Hanstrom, 1928, 1929; Graber, 1933; Walker, 1936; Stahl, 1938; de Lattin, 1939; Amar, 1950, 1951, 1953; Gabe, 1952; Miyawaki, 1958; Matsumoto, 1959 and Oguro, 1959a, 1959b, 1960, 1961). It is believed that the great structural variations are associated with the systematic position of the animals investigated. There is little information on the protocephalic neurosecretory pathway in Amphipoda and therefore, neurosecretory cells would not appear to have been investigated in this group except the identification of sinus gland in *Gammarus pulex; G. fluviatilis* and *G. locusta* (Graber, 1933; Stahl, 1938) and cephalic statocyst as an X-organ (Dahl, 1963). Being a primitive malacostracan, it would be interesting to compare the protocephalic neurosecretory pathway at one end with higher malacostracans and at the other end with primitive crustaceans. The present paper describes the structure of various types of neurosecretory cells found in the central nervous system of *Rivulogammarus syriacus* Chevreux, the distribution of these cells and the modes of secretion and discharge of the neurosecretory material in them.

MATERIAL AND METHODS

The freshwater *Rivulogammarus syriacus* was collected from the running water of Nowran in Mosul, Iraq. The average size of the female was 6.5 mm, ranging from 5.0 mm to 10.0 mm and of the male was 7.0 mm ranging from 5.0 mm to 11.0 mm. Whole specimens were fixed in aqueous Bouin, Helly and Carnoy fixatives. The anterior region was decapitated and after dehydration in alcohol and in xylene, was embedded in paraffin. Serial horizontal sections of 8 μ thickness were obtained. The following stainings and histochemical reactions were employed: (1) Bargmann's (1949) modification of Gomori chrome haematoxylin phloxine technique (CHP), (2) Gabe's (1953) modification of Gomori paraldehyde fuschin technique (PF), (3) Mallory's triple stain, (4) Heidenhain's Azan according to Hubschman (1962), (5) Ehrlich's haematoxylin and eosin (HE).

Rivulogammarus syriacus is a common inhabitant of Nowran stream, found all round the year underneath the stones and sometimes adhered to the algal and other aquatic plants. Their distribution is confined to the areas where the water is shallow and the current is slow. In spring and autumn, the frequency of *Rivulogammarus* is maximum and females and males have been seen in pairs. During these seasons the females carry eggs and juveniles. It indicates that *Rivulogammarus* has two breeding seasons in a year. They are inactive in winter as well as in summer.

RESULTS

Nervous system

The central nervous system of *Rivulogammarus syriacus* consists of a supraesophageal ganglion (brain), circumesophageal commissures, leading to the subesophageal ganglia and a ventral nerve cord. The brain, a vertical mass lies dorsad of the alimentary canal at the anterior surface of the head capsule, bearing dorsolaterally and ventrally two large pair of lobes, is divided into protocerebrum, deutocerebrum and tritocerebrum. These morphological divisions are obscured externally due to the reduction of the deutocerebrum. The protocerebrum is well developed and its dorsolateral lobe tapers towards the optic nerve (Fig. 1). The distal three-fourths of the dorsolateral lobe may be designated as optic lobe, though they are not demarcated by a peduncle as in many decapods. The deutocerebrum is not well developed in comparison with protocerebrum. The tritocerebrum tapers laterally, then posteriorly.

Types of nerve cells and their location

The neurosecretory cells of *Rivulogammarus syriacus* are distinguished into six types on the basis of size, general shape of the cell body, presence or absence of vacuoles in the cytoplasm, and staining properties of the secretory material and have been designated as types: A, B, B′, C, D, and E. (Figs. 2–13) in the present account. These cells exhibit definite localizations and are distributed in several groups in the peripheral region throughout the nervous system. These groups on the dorsal surface of the brain are: medio-dorsal, antero-dorsal, antero-dorso-lateral, dorso-lateral, postero-dorsal, and on the ventral surface are: antero-ventral, ventro-lateral, tritocerebrum, circumesophageal and ventral nerve chain (Fig. 1).

Structure of neurosecretory cells

A cell (*Figs. 2 and 8*). A cells are pyriform and with axons, measure about 15–20 μ in diameter. The cytoplasm has a dense granulated appearance and is full of mitochondria and Nissl's bodies. Minute secretory granules which are stained dark blue with CHP appear in the cytoplasm. These granules usually occur in aggregates just outside the Nissl zone. In addition, aggregates are often present at the extreme periphery of the cells. Most of these secretory granules are discharged from the perikaryon to the tissue fluid. Some other granules are sent into the axons. The direct relation between the granules and the nucleus is never observed in *Rivulogammarus syriacus*. The nucleolus and other chromatin like basophilic elements are present in the oval nuclei (7–10 μ in diameter) of these cells. The single nucleolus is large, basophilic and invariably found in the peripheral region of the nucleus. These cells are found in all the regions of the nervous system except in dorso-lateral and in the ventral nerve chain groups.

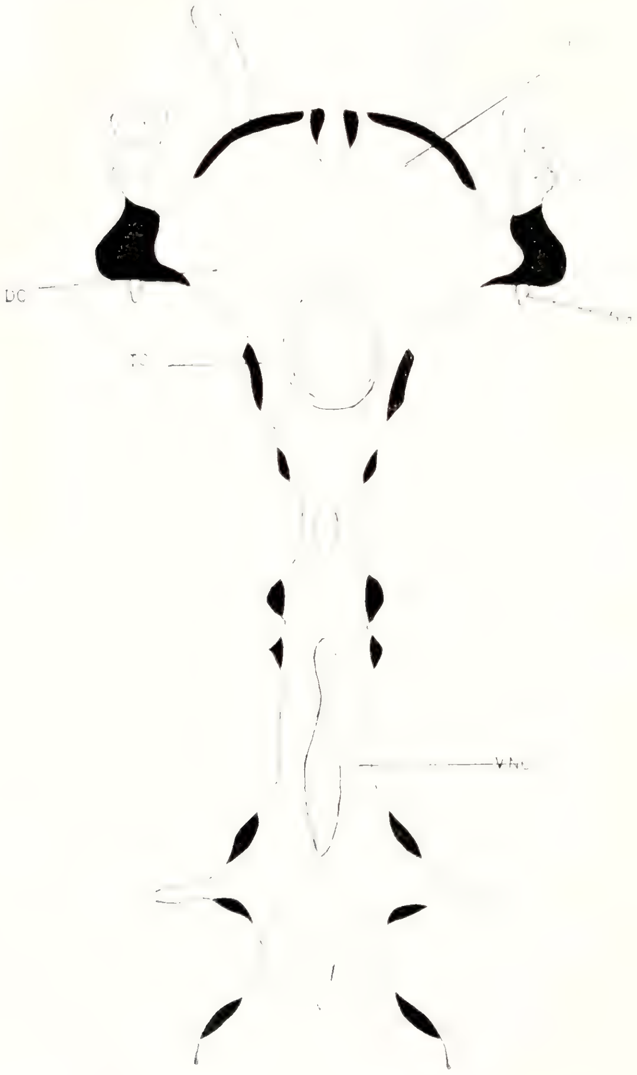
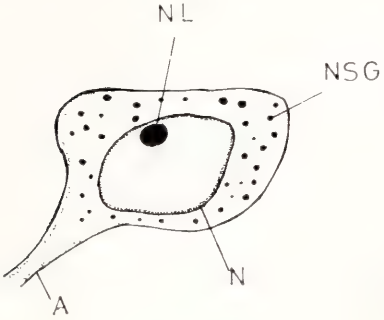


FIGURE 1. Dorsal plan of the central nervous system of *Rivulogammarus syriacus* Chevreux: Black areas show position of neurosecretory cells; PC—Protocerebrum; DC—Deutocerebrum; TC—Tritocerebrum; E—Eye; SG—Sinus gland; VNC—Ventral nerve cord.

B cell (Figs. 4 and 9). B cells, about 15 μ in diameter, are oval in shape and are characterized by the extensive granulation of the cytoplasm. The dark blue granules (CHP-positive and PF-negative) are scattered in the cytoplasm and



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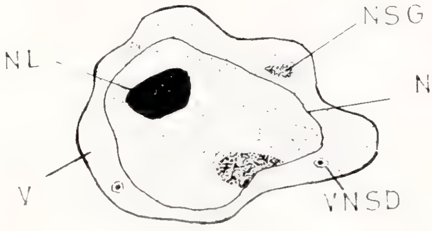

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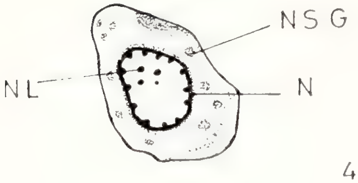

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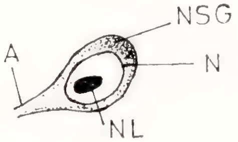

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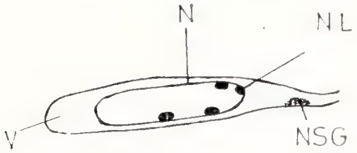

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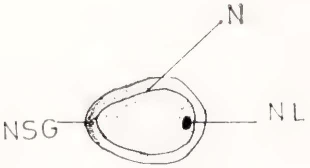

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FIGURE 2. Camera lucida drawing of A type of neurosecretory cell of *Rivulogammarus syriacus* Chevreux; 12X100; A—Axon; N—Nucleus; NL—Nucleolus; NSG—Neurosecretory granules.

FIGURE 3. Camera lucida drawing of B′ type of neurosecretory cell of *Rivulogammarus syriacus* Chevreux; 12X100; N—Nucleus; NL—Nucleolus; NSG—Neurosecretory granules; V—Vacuole; VNSD—Vacuole containing neurosecretory droplet.

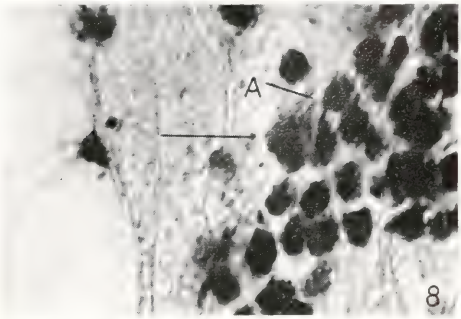
FIGURE 4. Camera lucida drawing of B type of neurosecretory cell of *Rivulogammarus syriacus* Chevreux; 12X100; N—Nucleus; NL—Nucleolus; NSG—Neurosecretory granules.

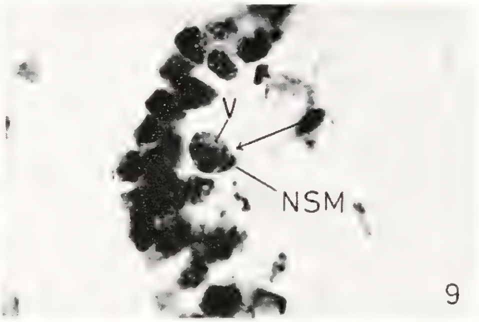
FIGURE 5. Camera lucida drawing of C type of neurosecretory cell of *Rivulogammarus syriacus* Chevreux; 12X100; A—Axon; N—Nucleus; NL—Nucleolus; NSG—Neurosecretory granules.

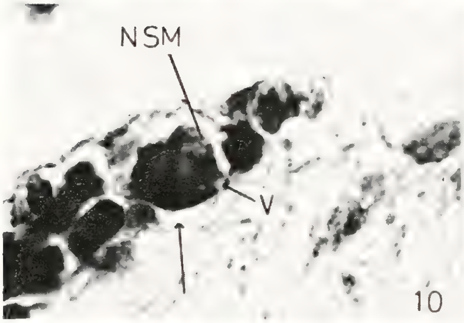
FIGURE 6. Camera lucida drawing of D type of neurosecretory cell of *Rivulogammarus syriacus* Chevreux; 12X100; N—Nucleus; NL—Nucleolus; NSG—Neurosecretory granules; V—Vacuole.

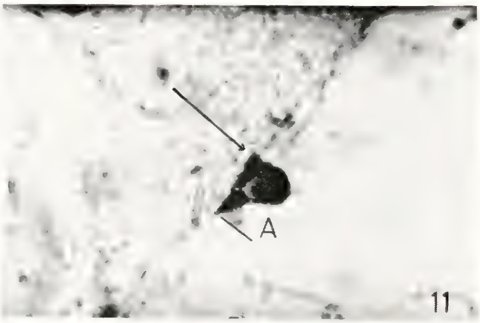
FIGURE 7. Camera lucida drawing of E type of neurosecretory cell of *Rivulogammarus syriacus* Chevreux; 12X100; N—Nucleus; NL—Nucleolus; NSG—Neurosecretory granules.

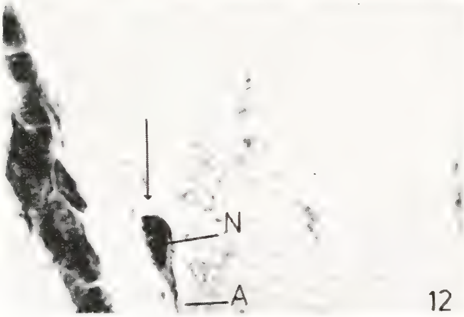
sometimes they are gathered at one side of the cell and form one or two relatively large masses. Frequently, the large mass of granules disappear from the cell and leaves behind a large vacuole. In most of these cells, the granules form a ring











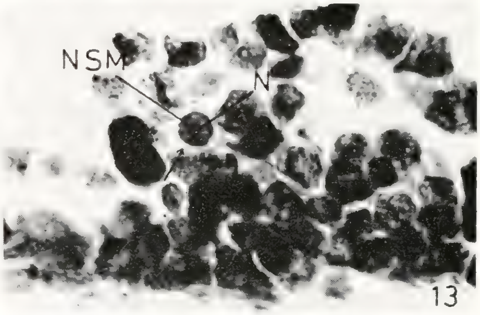


FIGURE 8. Horizontal section through the brain of *Rivulogammarus syriacus* Chevreux, Bouin, CHP. Note A type of cell (arrow), A—Axon.

FIGURE 9. Horizontal section through the brain of *Rivulogammarus syriacus* Chevreux, Bouin, CHP. Note B type of cell (arrow); NSM—Neurosecretory material; V—Vacuole.

FIGURE 10. Horizontal section through the brain of *Rivulogammarus syriacus* Chevreux, Bouin, CHP. Note B′ type of cell (arrow); NSM—Neurosecretory material; V—Vacuole.

FIGURE 11. Horizontal section through the brain of *Rivulogammarus syriacus,* Bouin, CHP. Note C type of cell (arrow); A—Axon.

FIGURE 12. Horizontal section through the brain of *Rivulogammarus syriacus* Chevreux, Bouin, CHP. Note D type of cell (arrow); A—Axon; N—Nucleus.

FIGURE 13. Horizontal section through the brain of *Rivulogammarus syriacus* Chevreux, Bouin, CHP. Note E type of cell (arrow); N—Nucleus; NSM—Neurosecretory material.

outside the nucleus in the vicinity of the Nissl zone. The secretory products are discharged into the tissue fluid directly from the periphery of the cell. The nucleus is distended spherically and measures 5 μ in diameter. It has a densely staining basophilic membrane. The nucleus consists of many peripheral nucleoli lying against the nuclear membrane. The cells are found in all the regions of the nervous system.

B′ cell (Figs. 3 and 10). B′ cells are large, 20–25 μ in diameter, and possess little vacuolated cytoplasm. These cells have irregular outlines which are likely caused by shrinkage during fixation. The dense deep stained cytoplasm contains many vacuoles especially at the periphery. The vacuole formation does not have any intimate correlation with nuclear modification. The contents of the vacuoles are found to be CHP- and PF-positive and are discharged directly into the tissue fluid. The empty vacuoles are found in the peripheral region of the cytoplasm. The distended spherical nucleus (12 μ in diameter) has a large basophilic nucleolus and acidophilic nucleoplasm. These cells are found only in the medio-dorsal region of the brain.

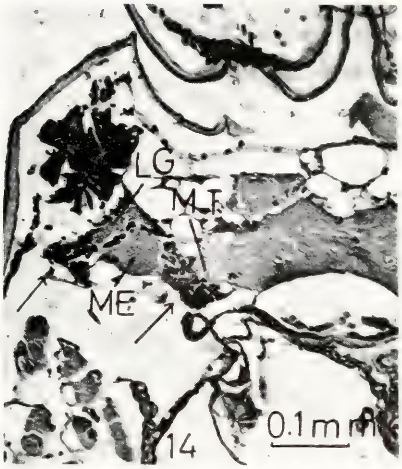
C cell (Figs. 5 and 11). C cells are small with thick axons and measure about 6–10 μ in diameter. They possess round nuclei 3–6 μ in diameter. The cytoplasm lacks vacuolation and presents a coarse appearance. Irregular masses of dark granules (CHP- and PF-positive) lie scattered around the nucleus. These granules have only one nucleolus. These cells are found in all the groups of the nervous system.

D cell (Figs. 6 and 12). D cells are cylindrical, measure 15–20 μ in diameter and have cylindrical nuclei, 8–10 μ in diameter. The deep stained cytoplasm contains a few vacuoles at the periphery. The contents of the vacuoles are found to be CHP-positive and PF-negative. The empty vacuoles are found to be arranged in form of a star. The nucleus contains several nucleoli. These cells are found only in medio-dorsal, antero-lateral and antero-ventral groups of the nervous system.

E cell (Figs. 7 and 13). E cells are oval in shape, 8–10 μ in diameter and possess little cytoplasm. The dark blue granules (CHP-positive and PF-negative) are scattered in the cytoplasm and sometimes are found in masses. Furthermore, all the cells do not show the presence of large amount of secretory material at a time. Apparently some cells are at the peak of their secretory process while others are devoid of granules and are in resting stage. The nucleus is round or oval (5–8 μ in diameter) and contains one nucleolus. These cells are found in all the groups of the nervous system.

Neurosecretory cells in the optic lobe

The optic lobe consists of lamina ganglionaris, medulla externa, medulla interna and medulla terminalis. The medulla interna is very poorly developed. There are two large groups of cells found all over the medulla externa and lamina ganglionaris. Since medulla terminalis is not demarcated from the protocerebrum, the neurosecretory cells of both these regions are not differentiated. The neurosecretory cells which are of E type form the anterior and posterior boundaries, 3–4 layers thick (Fig. 14). The X-organ is absent.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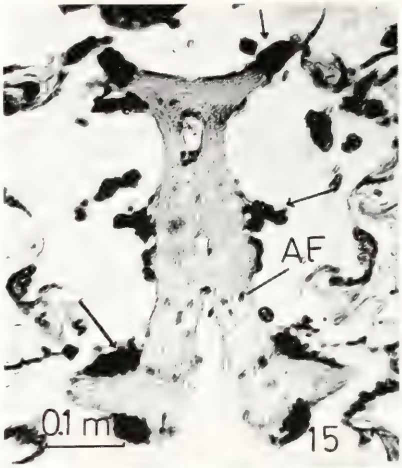

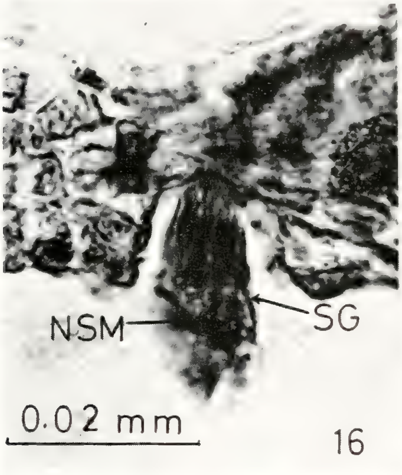
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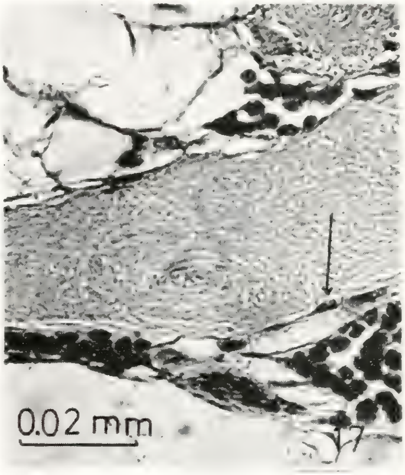


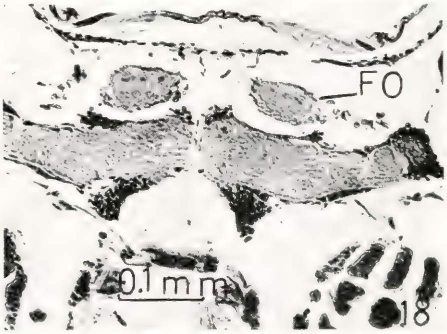


FIGURE 14. Horizontal section through the cephalic region of *Rivulogammarus syriacus* Chevreux showing parts of optic region. Note the various neurosecretory regions (arrows); Bouin, CHP; LG—Lamina ganglionaris; ME—Medulla externa; MT—Medulla terminalis.

Sinus gland

Sinus gland is an oval mass attached by a narrow stalk to the lateral tip and the distal part of the optic lobe. Its smooth capsule is continuous with that of optic lobe, and there are no attachments by blood vessels or connective tissue to any organ except the optic lobe. The sinus gland has a function of storage and release of neurosecretory products, which are elaborated in other sites. It contains nerve endings as does that of decapods, and no nucleus is found in the tissues of the sinus gland. The contents of the sinus gland are stained markedly dark blue with CHP but sometimes acidophilic substances are also detected. With PAS and toluidine blue the contents of the sinus gland are stained pink and violet blue, respectively. It has been observed that the sinus gland has two distinct regions, the distal and proximal (Fig. 16).

Frontal organs

These paired spherical bodies lying anterior to the protocerebrum, are connected to the medulla terminalis of the optic lobe by an oblique nerve. Two kinds of structural elements are observed in the frontal organs. The first kind are the cells without neurosecretory material surrounded by small connective tissue cells and neurons; the second are small round colloidal concretions. The latter seems to be the secretory product of neurosecretory cells and have been transported through the axons (Fig. 18). The neurosecretory material in form of CHP-positive granules are also found along the course of the axons coming from the medulla terminalis of the optic lobe. These neurosecretory materials have PAS-positive reaction.

Neurosecretory activity of different cell types

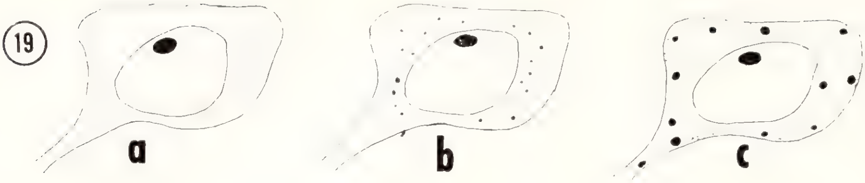
All the neurosecretory cells fall into two groups on the basis of their neurosecretory activities. The first group is comprised of A, C, and E types of cells. The first stage in the secretory cycle of these cells is marked by the dense granulation of entire cytoplasm and by appearance of small granular bodies within it (Fig. 19). These small granular bodies aggregate into larger and darker ones which are finally discharged either through the cell membrane into the tissue fluid or into the axons. The second group is comprised of B, B′, and D types of cells. The neurosecretory granules form a ring outside the Nissl zone (Fig. 20). The CHP-positive granules in B and D cells and CHP- and PF-positive granules in B′ cells, aggregate to form relatively large masses. Frequently the large masses of granules disappear from the cell and leave vacuoles behind (Fig. 10).

FIGURE 15. Horizontal section through the ventral nerve cord of *Rivulogammarus syriacus* Chevreux, Bouin, CHP. Note the various neurosecretory regions (arrows); AF—Axon fiber carrying neurosecretory material.

FIGURE 16. Horizontal section through cephalic region of *Rivulogammarus syriacus* Chevreux, Bouin, CHP. Note the sinus gland (arrow); NSM- Neurosecretory material; SG—Sinus gland.

FIGURE 17. Horizontal section through the cephalic region of *Rivulogammarus syriacus* Chevreux, Bouin, CHP. Note the axons carrying secretory material (arrow).

FIGURE 18. Horizontal section through cephalic region of *Rivulogammarus syriacus* Chevreux; Bouin, CHP; FO—Frontal organ.



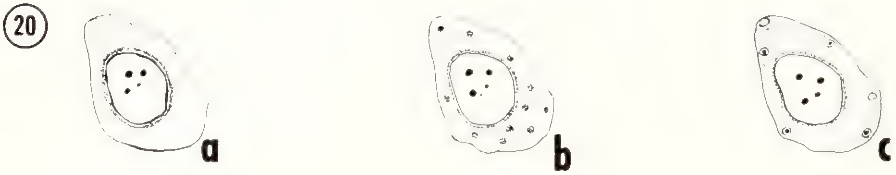


Figure 19. Various stages (A, B and C) in the secretory cycle of A type of cell; A—Entire granulation of cytoplasm and appearance of small granular bodies; B—Aggregation of small granular bodies into larger and darker ones; C—Presence of dark bodies in the axon and towards the periphery.

Figure 20. Various stages (A, B and C) in the secretory cycle of B type of cell; A—Neurosecretory granules form a ring outside the Nissl zone; B—Aggregation of neurosecretory granules into large masses; C—Release of large neurosecretory masses from the perikaryon and the appearance of vacuoles behind.

The migration of the neurosecretory products of these cells is accomplished in two ways: one is peripheral discharge from the perikaryon as observed in A, B, B′, and E types of cells; the other axonal transportation as seen in A, C, and D types. As is obvious, the migration of the secretory product in A type of cells can take place by both the methods (Fig. 19).

The peripheral discharged substances appear as dark-blue granules into the tissue fluid. These granules migrate with the tissue fluid and are stored in the spaces in a granular form. The axonally transported substances soon loses its identity and aggregate to form large masses. These masses are found in the axon fibers and are carried towards the optic lobes (Fig. 17).

The axon fibers run in the periphery of both anterior and posterior sides of the brain and finally end in the sinus gland. The axons coming from the anterior

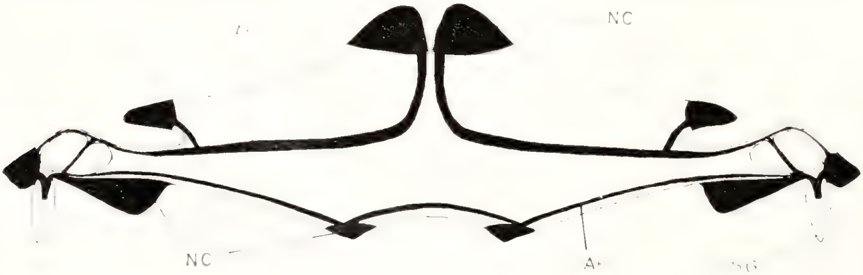


Figure 21. Diagrammatic horizontal section of the brain of *Rivulogammarus syriacus* Chevreux showing axons leading towards the sinus gland; AF—Axon fiber; NC—Neurosecretory cells; SG—Sinus gland.

side pass over the medulla externa and then bifurcate into two; one bundle makes a course between the medulla externa and lamina ganglionaris and finally ends in the sinus gland, the other passes over the lamina ganglionaris and then to the sinus gland (Fig. 21).

The neurosecretory axons in the ventral nerve cord can be traced by means of their pink color. The axons which run straight, carry the neurosecretory masses to the brain via circumesophageal connectives (Fig. 15).

Discussion

The neurosecretory cells of the crustaceans have been classified from a morphological point of view by several workers into many types. Enami (1951) described α, β, and γ types of cells in *Sesarma;* Matsumoto reported A, B, C, and D types of cells in *Eriocheir* (1954) and *Chionectes* (1956) and Inoue (1957) observed A, A′, B, C, D, and E types of cells in *Pachigrapsus.* Later Matsumoto (1958) considered eleven types of cells in *Potamon, Chionectes, Neptuns* and *Sesarma* but Miyawaki (1960) classified the neurosecretory cells of the decapod crustacea into three types: the giant, the medium and the small. Matsumoto (1959) reported four kinds of neurosecretory cells (A, B, B′, and Y) in an Isopod, *Armadillidium.* As regards Anostraca, Menon (1962) reported A and B types of cells in *Streptocephalus* sp.; Baid and Ramaswamy (1965) observed A and B cells in *Artemia salina* and Hentschel (1965) described U-1, U-2, and U-3 cells in *Chirocephalus* and *Artemia salina.* Recently Lake (1969) observed small cerebral PF-positive monopolar cells, cerebral bipolar PF-positive cells and medium PF-positive in *Chirocephalus diaphanus.*

A histological examination of the central nervous system of *Rivulogammarus syriacus* shows the presence of six kinds of neurosecretory cells (A, B, B′, C, D. and E types). The size of the cells, the mode of discharge of their secretory product and the nature of the secretory granules have formed the basis of such classification.

The morphological characteristics of these cells except A and E types are quite different from that of decapod crustaceans. A type cells of *Rivulogammarus,* judged from their size, would seem to correspond to A cells of *Potamon* (Matumoto, 1958; Baid, Hafidh and Dabbagh, 1968), Enami's (1951) α cells and A cells of *Pachigrapsus* (Inoue, 1957) but the other morphological characteristics of these two cells are also quite different. The nucleus of A cells of these authors show polymorphic appearance and such a condition is not found in A cells of *Rivulogammarus.* The A cells of *Rivulogammarus* correspond to large cells of *Idotea* (Matumoto, 1958) and to A cells of *Armadillidium* (Matumoto, 1959). The E cells of *Rivulogammarus* are quite similar to B cells of other crustaceans (Enami, 1951; Matumoto, 1954, 1958; Inoue, 1957 and Baid, Hafidh and Dabbagh, 1968) concerning their size, shape and structure and stainability of their granules. They are the commonest neurosecretory cells in crustacea. The characteristics of other kinds of cells (B, B′, C, and D) are somewhat different from those of the similarly named cells in crabs. The D types of cells are very distinct, conspicuous and as far as we know, have not been reported earlier.

In entomostracans, the neurosecretory cells have been reported in Cirripedia by Barnes and Gonor (1958), in Cladocera by Sterba (1957), Angel (1966) and

Parker (1966) and in Anostraca by Menon (1962), Baid and Ramaswamy (1965), Hentschel (1965) and Lake (1969). The neurosecretory cells of *Rivulogammarus* do not correspond to any of the cells reported in entomostracans.

The neurosecretory products elaborated in the cytoplasm of the neurosecretory cells, have been studied morphologically by many authors by the use of usual histological techniques (Gomori-chrome-alum haematoxylin phloxine method) and more specialized methods in both vertebrates and invertebrates. Chrome-haematoxylin stained substances (Bliss, Durand and Welsh, 1954), phloxinophilic substances (Matsumoto, 1958), acid fuschin stained substance (Enami, 1951), PAS-positive material (Miyawaki, 1956, 1958) and many other materials stained by various methods have been reported as neurosecretory products. All the cells in the present study are positive to CHP but are negative to PF except B′ and C cells.

Concerning the distribution of neurosecretory cells, A cells are confined to the brain but B, C, and E cells are found both in the brain and the ventral nerve cord ganglia. In the optic lobe of *Rivulogammarus* only E cells are found while in *Armadillidium* Y cells are present (Matumoto, 1959). It is difficult to say whether E cells of *Rivulogammarus* correspond to Y cells of *Armadillidium* (Isopoda). In crabs many kinds of neurosecretory cells are found in the optic lobe but B cells are confined to the ventral corner of the medulla terminalis. In *Armadillium,* they are situated at the anterior side of the brain and at the base of the optic lobe peduncle. The E cells of *Rivulogammarus,* similar to B cells of other crustaceans are distributed in all the regions including the optic lobe. The location of B cells may be considered to show some resemblance in the different orders of Crustacea.

The discharge of neurosecretory substances was earlier believed to take place in two possible ways (Scharrer and Scharrer, 1945; Matumoto, 1958); to these has been added a third by Matumoto (1958). These possibilities are into the tissue spaces of the ganglion, the pedal nerves or the circumesophageal commissures. The axonal transportation of neurosecretory substances has been confirmed by several investigators in the recent past. These observations have contributed to the evolution of the concept of a neurosecretory system in decapod crustacea parallel to the hypothalamic-hypophyseal system of the vertebrates (Bargmann, 1957) and to the inter-cerebralis-cardiacum-allatum system of insects (Scharrer and Scharrer, 1945; Scharrer, 1952). Enami (1951) suggested an axonal transport of neurosecretory material; Bliss and Welsh (1952) came to the conclusion that the neurosecretory substances produced in various parts of the central nervous system migrate along the nerve fibers to the sinus gland where they are stored and eventually released. Matumoto (1954) reported the release of neurosecretory substances in the network of capillaries which closely surround the neurosecretory cells. In the present study, it has been found that the neurosecretory substances are axonally transported and are stored in tissue spaces. The tracing of the pink axon shows that the neurosecretory products of the ventral nerve chain ganglia are transported mainly to the circumesophageal commissure. Further, they are carried to the brain and then to the eyestalks. A similar observation has been reported by Bliss, Durand and Welsh (1954).

The sinus gland in *Gammarus* was described by Graber (1933) as pseudofrontal organ and by Stahl (1938). Oguro (1959a, 1959b) reported two pairs of sinus glands in three idotead species and in (1960) described a sinus gland in addition to a pseudofrontal organ in *Tecticeps*. Walker (1936) reported that the pseudofrontal organ described might be identical with the white organ (Koller, 1930) and haemal gland (Hanstrom, 1933) both of which are now designated as sinus gland. Gabe (1952) considered that the pseudofrontal organ is identical with the sinus gland in *Oniscus asellus*. Amar (1950, 1953) stated that the pseudofrontal organ in isopods may be homologous with the sinus gland. Recently, Fingerman (1956) described that with respect to location and structure, the sinus gland of *Ligia exotica* is similar to the organ described by Walker (1936) for *Oniscus asellus*. In *Rivulogammarus* the sinus gland is found to be identical in position and structure to the pseudofrontal organ described by Graber (1933) in *Gammarus*. The sinus gland of this amphipod has two distinct regions: the proximal and the distal. The occurrence of distinct zones, with different tinctorial qualities in the sinus glands of malacostracans has been reported by Enami (1951); Potter (1958) and Carlisle (1959).

There has been a considerable amount of discussion concerning the nomenclature and function of the organs of Bellonci and frontal organs. Claus (1886) described the dorsal frontal organs and regarded them as sensory structures. Hanstrom (1931, 1934) described the frontal organs in *Tanymastix stagnalis* L. and *Polyartemia forcipata* and later (1937), attributed a secretory function to them and regarded them to be precursors of the X-organs of malacostracans. Elofsson (1963) working with different decapods, was able to show that the dorsal frontal organs when present, are always part of the nauplius eye center of the brain. The organs of Bellonci (sensory pore X-organs) are associated with the medulla terminalis in the Decapoda (Knowles and Carlisle, 1956) and have been shown by Dahl (1957) to be derived from neuroblasts of the medulla terminalis. Dahl (1959) has concluded that the dorsal frontal organs and the organs of Bellonci represent phylogenetically quite independent structures because the dorsal frontal organs in the crustacea investigated so far, when present, are associated with the nauplius eye center, whilst the organs of Bellonci are associated with the medulla terminalis. Menon (1962), Elofsson (1966) and Lake (1969) have attributed neurosecretory function to the organs of Bellonci (dorsal front organs) in Anostraca.

The paired frontal organs have been described by Thore (1932) and Hanstrom (1947) in *Gammarus* and with reservations in *Caprella*. According to Graber (1933), these organs do not exist in *Gammarus* and thus those described by others constitute something else than frontal organs. In *Rivulogammarus syriacus* the paired frontal organs are quite distinct and are not innervated from the nauplius eye center but from the medulla terminalis. These paired structures and their axons contain appreciable amount of CHP-positive granules. It appears that these structures are associated with both neurosecretory release and perhaps neurosecretory material synthesis. These functions are similar to those of organs of Bellonci of other malacostracans, the frontal organs of the Copepoda (Carlisle and Pitman, 1961), the X-organs of the Copepoda (Elofsson, 1966), the sensory papilla X-organ of cirriped larvae (Kauri, 1966), and finally to the dorsal frontal organs or X-organs of the Anostraca (Hanstrom, 1934; Menon, 1962 and Elofsson, 1966).

The physiological significance of these six types of neurosecretory cells in central nervous system of *Rivulogammarus syriacus* is yet unknown. As reported in other crustaceans, possibly some of the neurosecretory cells may also be the source of chromatophorotropic principle. It will be interesting to investigate the physiological significance of the different neurosecretory cells in *Rivulogammarus syriacus.*

SUMMARY

1. The different types of neurosecretory cells occurring in the central nervous system of the freshwater amphipod, *Rivulogammarus syriacus,* their distribution, neurosecretory activity, and the mode of discharge of the neurosecretory substances were studied.

2. There are six types of neurosecretory cells designated as A, B, B′, C, D and E which show definite localization in the central nervous system. The B′ cells are found only in the medio-dorsal region.

3. These cells can be divided into two groups on the basis of their secretory activities.

(a) The first group of cells (A, C and E types) have dense granulated cytoplasm marked by small granular bodies. These bodies aggregate into larger and darker stained ones and are finally discharged either through the cell membrane into the tissue fluid or into the axons.

(b) The second group of cells (B, B′ and D types) have neurosecretory granules in the form of a ring outside the Nissl zone. The granules aggregate to form large masses which disappear through the cell and leave vacuoles behind.

4. The contents of the sinus gland show CHP-positive, PAS-positive, toluidine blue positive and sometimes acidophilic substances.

5. It appears that the frontal organs are associated with neurosecretory release.

6. The physiological activities of these neurosecretory substances in the central nervous system of *Rivulogammarus* are as yet unknown.

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COMPARATIVE FERTILITY OF GAMETES FROM SIX SPECIES OF SEA URCHINS

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Gametes from marine animals are readily fertilized *in vitro* and fertility can, therefore, be defined and investigated quantitatively, in terms of the success of gamete interaction. Sea urchin gametes are particularly suitable for such analysis because of the ease of obtaining gametes and fertilization under controllable conditions (*cf.* Tyler and Tyler, 1966a).

Several early investigators tried to determine the minimum number of sperm necessary to activate an egg (*cf.* Glasser, 1915 and Lillie, 1915). Their results were inconclusive because they evaluated sperm concentration as dilution of "dry sperm" (*i.e.*, the thick, undiluted semen exuded from the animal) rather than numbers of sperm, and defined fertility in terms of the percentage of eggs fertilized. Such research, although not truly quantitative, set precedents which have been followed in most subsequent studies (*cf.* Tyler and Tyler, 1966a, 1966b).

The objective of the research reported here was to compare the fertility of gametes from different species of sea urchins. A method was devised for determining the ratio of spermatozoons/fertilization under defined conditions. The procedure was standardized so that dilution effects and the subsequent rapid aging of sperm were minimized (*cf.* Branham, 1966). A suitable working definition of *in vitro* fertility was devised by examining the results of numerous crosses. The data were then evaluated statistically to compare the various species.

MATERIAL AND METHODS

Six species of regular echinoids with convenient breeding seasons are readily available around Oahu in Hawaii (Table I). Gametes were obtained from them by injecting isotonic KCl (0.53 M) into the coelomic cavity during the seasons indicated in Table I.

Injected females were inverted over a beaker so that eggs were shed directly into sea water. The eggs were washed with several changes of sea water and their concentration determined by counting the eggs in a portion of the stock suspension. Males were also inverted over a beaker and semen shed directly into sea water acidified to pH 7.0 with carbon dioxide. Under these conditions the sperm were relatively inactive and the semen accumulated as a layer on the bottom of the beaker (*cf.* Branham, 1966). Stock sperm suspensions were prepared by stirring a small volume of concentrated semen into additional acidified sea water until the desired concentration was obtained.

Sperm concentration was calculated from the optical density (O.D.) of the stock suspension determined with a Klett Summerson colorimeter (Green filter, 520–580 mμ), by multiplying O.D. by a species specific constant (see Table I).

The constants were calculated by comparing sperm concentration determined by haemocytometer counts, with the O.D., for at least one hundred sperm suspensions for each species.

Fertility titrations were carried out in a series of "wells" in plexiglas blocks ("trays") arranged so that the gametes could be viewed from below with an inverted microscope. The wells were 11 mm deep and had end areas of one cm^2. Each tray contained a single row of ten wells. Eppendorf automatic microliter pipettes with disposable tips were used to fill the wells and prepare serial dilutions of semen.

The standard procedure was to make a ten step, two-fold dilution series of the stock sperm suspension of known optical density and then add approximately 200 eggs to each well. One half ml of stock was added to 0.5 ml of sea water and one half of this dilution transferred into 0.5 ml of sea water in the next well, and so on. This procedure took less than one minute per tray. The proportion of eggs with fertilization membranes was determined by examining one hundred eggs in each well, beginning at least 20 minutes after insemination. All eggs were counted in three wells of each tray and the average number of eggs/well in that tray calculated. Sixteen trays with a total of 160 wells were used, so that four sperm suspensions could be crossed with four egg suspensions in all possible combinations. The optical density of the stock sperm suspension, the volume, the average number of eggs and the percentage of eggs fertilized in each well were recorded on IBM cards and analyzed statistically with the aid of an IBM 360 computer.

EXPERIMENTS AND RESULTS

In order to evaluate the relative fertility of gametes from different sea urchins it was necessary to standardize a quantitative method for determining fertility. This has customarily been attempted by preparing a serial dilution of semen (expressed as some fraction of "dry" semen), adding eggs and determining the percentage of eggs fertilized in each sperm dilution step, or arbitrarily choosing as an end point the dilution below which less than some given percentage of fertilization occurred (*cf.* Tyler and Tyler, 1966b). This procedure was modified in the following experiments, so that the approximate number of gametes could be determined and dilution effects and sperm aging could be minimized.

Lillie (1915) observed that the sequence of mixing gametes influenced such fertility titrations. He noted that higher percentages of fertilization were obtained in greater dilutions of semen when sperm suspensions were added to eggs rather than vice versa. He demonstrated that this was the result of an effect of time after sperm dilution, which varied with the sequence used. Such aging of sperm can be controlled by keeping the sperm immobilized in acidified sea water (Branham, 1966).

Carbon dioxide, added to sea water until a pH of 7.0 was obtained, reduced the motility of sperm from the six Hawaiian species. The fertility of such immobilized sperm was compared with that of normally active sperm in the following titration experiments. "Dry" *T. gratilla* semen was diluted in sea water (pH 8.2) or CO_2 charged sea water (pH 7.0) and the optical density measured. The suspensions were serially diluted with sea water (pH 8.2) after about ten minutes and eggs added. Sperm in CO_2 sea water were at least six, and usually eight or more, dilution steps (2^6–2^8) more "fertile" than the controls.

The repeatability of such determinations was examined in two experiments with *T. gratilla* gametes. In each experiment a pH 7.0 sperm suspension was prepared which gave about 50% fertilization after $\frac{1}{2}$ dilution in sea water. This was then transferred as quickly as possible (beginning within five minutes of initial dilution) in 0.5 ml portions into 0.5 ml of pH 8.2 sea water in each of the 160 wells of the sixteen trays. Eggs were added to each tray as it was filled (about one minute after sperm were added to the first well in each tray) and the last well received eggs 18 minutes after the first one. In both experiments the percentage of eggs fertilized in each well was the same within an 8% range and the last wells filled were not significantly different from the first ones. In two control experiments with normal sea water (pH 8.0) following the same time schedule, considerably higher percentages of eggs were activated in the first wells than in the last ones.

TABLE I

Species of sea urchins examined

Genus and species	Order and family	Season when "ripe"	Sperm concentration constants
	Order: Echinoida		
Colobocentrotus atratus	Family: Echinometridae	Sept.-July	$4.3\ 8 \times 10^7$
Echinometra mathaei	Family: Echinometridae	Sept.–May	$5.3\ 7 \times 10^7$
Echinometra oblonga	Family: Echinometridae	Sept.–May	$4.9\ 4 \times 10^7$
Heterocentrotus mammillatus	Family: Echinometridae	Sept.–May	$5.9\ 7 \times 10^7$
	Order: Temnopleuroida		
Pseudoboletia indiana	Family: Toxopneustidae	All year	$4.7\ 7 \times 10^7$
Tripneustes gratilla	Family: Toxopneustidae	All year	$3.6\ 7 \times 10^7$

These results indicated that variability due to sperm aging was important but could be controlled, and therefore, in all subsequent experiments sperm were initially diluted in acidified sea water.

Procedures of adding CO_2 immobilized sperm to eggs or eggs to sperm were compared in three experiments with *T. gratilla* gametes. The results were equivalent with both procedures but there were fewer manipulative steps involved when eggs were added to serially diluted semen so this sequence was used in subsequent titrations. The procedure is outlined in the Materials and Methods section.

Many crosses were made with this procedure and the results evaluated to produce a working definition of gamete fertility. The results of selected titrations are presented in Figure 1. The examples were selected at random from various categories of data that became apparent as the analysis progressed. *T. gratilla* gametes were on the average, about the most fertile, while *P. indiana* required the largest number of sperm/fertilization; *E. mathaei* and *E. oblonga* were chosen because they represented two species of the same genus. The remaining two graphs were chosen as representative of crosses between different species.

In the determinations in Figure 1, stock semen suspensions were prepared in CO_2 acidified sea water (pH 7.0) and their O.D. determined with a Klett Summerson colorimeter. The stock suspension was then diluted in filtered sea water in 10 two-fold steps, beginning with a $\frac{1}{2}$ dilution so that the numbers on the *abscissa* are step numbers or the negative Log_2 of the stock sperm dilutions. One drop containing about 200 eggs was added to each 0.5 ml semen dilution. The percentage of eggs with membranes was determined after 20 minutes. Sperm/fertilization was calculated in each well by the formula: (O.D. × sperm constant × Volume × dilution factor)/(% Fertilization × Average number of eggs per well).

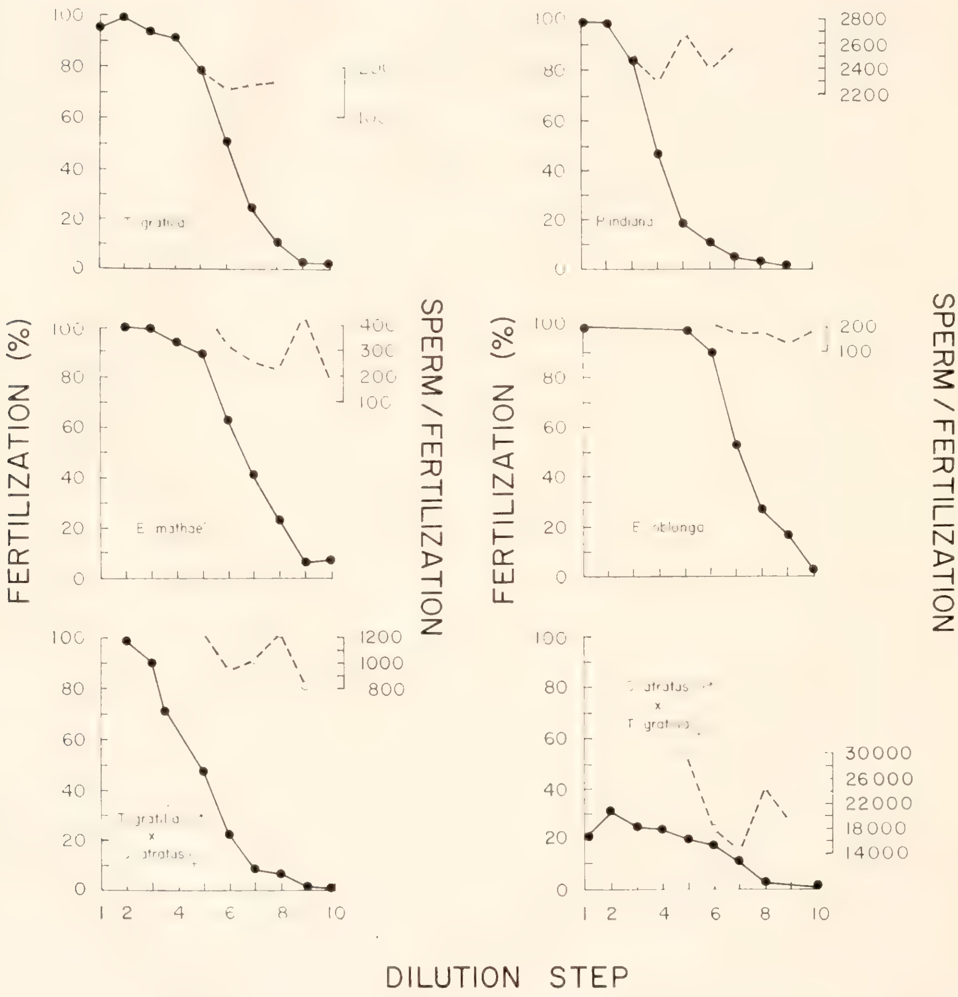

FIGURE 1. Representative titration results, comparing percentage of eggs fertilized with the number of sperm/fertilization.

In each case the first few wells contained an excess of spermatozoa and the maximum percentage of eggs were fertilized (percentage fertilized, solid line, scale on left ordinate) but in subsequent two-fold dilutions of semen the number of spermatozoa became limiting and the percentage of eggs fertilized declined. The ratio of total number of sperm to the calculated number of eggs fertilized (dashed line, scale on the right ordinate) remained fairly constant in those wells wherein less than 91 per cent and more than 3 per cent of the eggs raised fertilization membranes. Within these limits the percentage (and also total number) of eggs fertilized declined by approximately one half for each two-fold semen dilution (in ordinary unacidified sea water, this was not so; the decline in fertilization was more rapid, *cf.* Lillie, 1915, Figure 2). The ratio of sperm/fertilization within the prescribed limits was probably characteristic of the particular cross and was used for subsequent analysis. It or its reciprocal (fertilizations/sperm) could be used to define "gamete fertility" or "fertilizing capacity." In practice the values obtained from different wells varied somewhat, even within the chosen limits. The value characteristic of the cross was arrived at either by averaging the values that occurred within the limits or simply by using the lowest value obtained.

TABLE II

Analysis of within-species crosses

	Crosses (n)*	Average sperm/fertilization	SE†	% SE/AV	Smallest–largest sperm/fertilization
C. atratus					
	1 × 1 (16)	258	± 17	7%	178– 427
	4 × 4 (16)	116	± 14	12%	42– 235
	All (120)	631	±196	31%	14–19,010
E. mathaei					
	1 × 1 (16)	1,535	±107	7%	1,132– 2,145
	4 × (16)	658	±142	22%	254– 2,145
	All (69)	671	±109	16%	22– 5,034
E. oblonga					
	1 × 1 (16)	199	± 21	11%	135– 339
	4 × 4 (16)	265	± 47	18%	95– 670
	All (67)	356	±115	32%	21– 7,797
H. mammillatus					
	1 × 1 (16)	47	± 2	4%	32– 56
	4 × 4 (16)	64	± 8	12%	31– 139
	All (35)	112	± 15	14%	31– 398
P. indiana					
	1 × 1 (16)	2,002	± 96	5%	1,355– 2,469
	4 × 4 (16)	1,652	±406	25%	318– 5,962
	All (99)	3,542	±556	16%	218–42,766
T. gratilla					
	1 × 1 (16)	588	± 74	13%	87– 1,229
	4 × 4 (16)††	194	±100	52%	20– 1,602
	All (282)	285	± 42	15%	4– 8,048

* 1 × 1, gametes from one male and one female, subdivided into 4 semen and 4 egg suspensions which were then crossed in the 16 possible ways; 4 × 4, gametes from 4 males and 4 females crossed in 16 possible ways; *all* crosses between individuals of the same species wherein at least three wells contained 4 to 90% fertilization and there was no fertilization in the stock suspension of eggs.

† SE = Standard deviation /$\sqrt{n}$.

†† See Table III for the individual results of this series of crosses.

The data from crosses between individuals of the same species are summarized in Table II. The error inherent to the method was assayed several times for each species by making four separate egg suspensions from one female and crossing them in the sixteen possible ways with four separate sperm suspensions from a single male (1 × 1 crosses in Table II). The number of sperm per fertilization was calculated for each well and the results analyzed statistically in order to find the limits which yielded the greatest precision. The least variability, expressed as the ratio S.E./mean, in per cent, for the 16 determinations in each test resulted when the smallest value in each tray was selected for comparison with other trays. The variability was greater when average values, or the average of the three lowest values in each tray, was used for comparison, so the *smallest value in each tray was subsequently used for analysis.*

Gametes from four males and four females were also crossed in the 16 possible ways (4 × 4 crosses). The data were analyzed in the same way that the confidence limits were established, and selected examples are also presented in Table II. The examples selected for presentation were chosen at random from those experiments wherein each of the sixteen possible crosses was successful, *i.e.*, had at least three wells with more than 3% and less than 91% of the eggs fertilized, and none of the eggs in the stock suspension were fertilized. (In some experiments the stock sperm suspension contained too many or too few sperm and the cross could not be evaluated). In most cases the amount of variability was greater than that inherent to the method determined with repeated crosses of the same gametes (1 × 1). The results of the sixteen *T. gratilla* crosses selected for analysis in Table II are displayed in Table III. They are an extreme example.

TABLE III

The number of sperm/fertilization in the sixteen simultaneous crosses between four males and four female T. gratilla *presented in Table II*

	Males			
	1	2	3	4
Females				
1	27	44	120	41
2	44	20	1,602	106
3	20	68	150	100
4	60	20	601	75

The data from all successful crosses between individuals of the same species were pooled to examine possible species differences in gamete fertility (*all*, in Table II). The average should be viewed with caution because the data were not normally distributed. In each case kurtosis and skewness were significantly greater than normal, indicating that the distributions were flatter than normal and skewed to the right (Table IV). One interpretation of the observed distributions is that some proportion of the crosses were abnormally infertile, *i.e.*, required larger numbers of sperm/fertilization than normal, and that these constituted the tail of the distribution. This possibility was examined by successively excluding an increasing proportion of these highest values from each population until the distribution no longer deviated significantly from normal kurtosis and skewness ($P < 0.001$). The proportion of "abnormally" high values excluded varied for each species, as seen in Table IV.

The data from the various species were compared statistically. Such comparisons should be viewed with caution because the data deviated from a normal distribution in some cases and because the variances of the data were heterogeneous. The results of an approximate test of equality of means with heterogeneous variances (described by Sokal and Rolf, 1969) indicated that there were significant differences between the means ($P < 0.001$). The probability of difference between means was therefore examined with a t-test matrix, in Table V, using all the data, log transformation of the data, or the values obtained after excluding the highest values, as described above.

TABLE IV

Analysis of distribution of all data and selected data exclusive of "abnormally" infertile crosses†

Species	All values					Values exclusive of highest ones					
	N	Skewness	Kurtosis	Sperm/fert.		Excluded %	N	Skewness	Kurtosis	Sperm/fert.	
				AV ± SE	Coe. var.††					AV ± SE	Coe. var.††
C. atratus	120	6.346***	46.670***	631 ± 196	3.40	20	96	0.442	−0.769	91 ± 5	0.58
E. mathaei	69	2.584***	7.636***	671 ± 109	1.36	30	48	0.279	−1.022	232 ± 21	0.63
E. oblonga	67	7.619***	58.226***	356 ± 115	2.64	15	57	0.190	−0.131	184 ± 10	0.42
H. mammillatus	35	1.548***	1.646*	112 ± 15	0.82	30	24	0.757	−0.082	60 ± 4	0.35
P. indiana	99	4.561***	26.077**	3542 ± 556	1.56	25	74	0.412	−0.941	1611 ± 116	0.62
T. gratilla	282	7.364***	66.673***	285 ± 42	2.48	55	127	0.415	−0.800	32 ± 2	0.57

† Skewness and kurtosis were computed, and their deviation from normal values evluated by t-Test, as described by Sokal and Rohlf (1969). The highest values were sequentially eliminated from the analysis, in increments of 5% of N, until both values no longer deviated significantly ($P < 0.05$) from normal.

†† Coefficient of variation = s.d./mean.

* $P < 0.050$, that statistic represents normally distributed data.

** $P < 0.01$, that statistic represents normally distributed data.

*** $P < 0.001$, that statistic represents normally distributed data.

A few crosses were made between species and the results, although too limited to be definitive, suggest a useful addition to thinking about cross fertilization (Table VI). In most cases the percentages of eggs activated were great enough to give titration curves comparable to within species crosses even though a large proportion of eggs were usually not cross activateable (see representative example in Fig. 1). The ratio of sperm/activation in these cases could be calculated as it was for within species crosses and remained fairly constant after the sperm concentration became limiting. This ratio, when considered along with the maximum proportion of eggs activatable by sperm of the other species (which varied greatly within each combination examined), and the ratio of successful cases (*i.e.* > 3% activation) to the number of trials, gave a quantitative estimation of cross reactivity that facilitated comparisons (Table VI).

DISCUSSION

The procedure gave a rough quantitative measure of fertility. It combined traditional estimates of fertility in terms of the percentage of eggs fertilized at various semen dilutions with estimates of the number of sperm present per fertilized egg under standardized conditions. Its main fault was its large inherent standard error of about 8% of the mean values (see Table II). This error probably resulted from imprecision in determining the percentage of eggs fertilized (as shown by preliminary experiments where values varied about 8% when repeated determinations were made with the same sperm and egg samples) and imprecision inherent in calculating the total number of fertilized eggs by multiplying the average number of eggs by the percentage fertilized. By hindsight, it would have been better to simply count all fertilized eggs in each well wherein less than 91 and more than 3% fertilization occurred, but that was not apparent until after the data were analyzed.

TABLE V

Probability of similarities between the mean values of sperm per fertilization of the six species, when the means were calculated from all values, the log of all values or the values remaining after the exclusion of a proportion of the highest values as indicated in Table IV

	E. mathaei	*E. oblonga*	*H. mammillatus*	*P. indiana*	*T. gratilla*
C. atratus					
All	>0.5	<0.4	<0.2	<0.001	<0.01
Log	<0.001	<0.025	<0.1	<0.001	<0.1
Less top values	<0.001	<0.001	<0.01	<0.001	<0.001
E. mathaei					
All		<0.05	<0.001	<0.001	<0.001
Log		<0.025	<0.001	<0.001	<0.001
Less top values		<0.05	<0.001	<0.001	<0.001
E. oblonga					
All			<0.02	<0.001	<0.4
Log			<0.001	<0.001	<0.001
Less top values			<0.001	<0.001	<0.001
H. mammillatus					
All				<0.001	<0.4
Log				<0.001	>0.5
Less top values				<0.001	<0.001
P. indiana					
All					<0.001
Log					<0.001
Less top values					<0.001

The variation found when gametes from four pairs of urchins were crossed simultaneously (4 × 4 = 16 crosses) was usually greater than that due to the error of the method as determined from 16 simultaneous replications of the same cross (Table II). Such variation could have resulted from differences in the

TABLE VI

Evaluation of within- and between- species crosses

Females	Males *C. atratus*	*E. mathaei*	*E. oblonga*	*H. mammillatus*	*P. indiana*	*T. gratilla*
C. atratus						
Sperm/activation ± SE	631 ± 196	49,935 ± 47,893	74,665 ± 55,396	Not done	4,146 ± 1,669	3,707 ± 1,292
Range	14 to 19,010	205 to 433,064	846 to 294,806		672 to 8,657	46 to 26,328
Max. % activation		99%	86%		99%	99%
Successful/trials		9/9	5/5		5/5	26/26
E. mathaei						
Sperm/activation ± SE	109,435 ± 25,732	670 ± 109	550,895 ± 378,582	Not done	294,398 ± 99,132	148,842 ± 68,538
Range	2,311 to 229,271	22 to 5,034	974, to 4,278,392		3,322 to 963,937	6,627 to 421,074
Max. % activation	32%		40%		57%	33%
Successful/trials	10/11		11/28		9/9	6/10
E. oblonga						
Sperm/activation ± SE	8,150	133,154 ± 67,689	356 ± 115	Not done	Not done	86,056
Range	772 to 15,528	106 to 1,261,949	21 to 7,798			416 to 171,966
Max. % activation	30%	90%				17%
Successful/trials	2/3	18/28				2/5
H. mammillatus						
Sperm/activation ± SE	Not done	Not done	Not done	112 ± 15	Not done	3,532 ± 1,714
Range				31 to 398		448 to 9,897
Max. % activation						35%
Successful/trials						5/5
P. indiana						
Sperm/activation ± SE	122,957 ± 59,910	613,155 ± 294,943	Not done	Not done	3,542 ± 556	—
Range	7,756 to 320,948	12,092 to 2,147,999			218 to 42,766	—
Max. % activation	43%	44%				1%
Successful/trials	5/5	7/11				0/12
T. gratilla						
Sperm/activation ± SE	76,756 ± 28,177	94,677 ± 27,890	23,963 ± 22,142	14,806	—	256 ± 42
Range	5,758 to 447,954	1,895 to 241,277	705 to 68,228		—	4 to 8,048
Max. % activation	31%	29%	60%	7%	1%	
Successful/trials	16/23	7/8	3/4	1/6	0/13	

fertilizing capacity of the sperm samples, differences in fertilizability of the egg samples or differences in the compatibility of gametes in each particular combination. Variation in sperm fertilizing capacity apparently dominated in these results, as can be seen from Table III wherein male three (and to a lesser extent four) stands out as less fertile than the others. Variations in the fertilizability of eggs (those from female one appear slightly more fertile) and particular combinations (male four and female one, male two and female three, male three and female two, and male four and female one seem to stand out) might also have had some effect.

Variation is also apparent from the large range of values obtained when all crosses within the same species are considered. Such values were not normally distributed in most cases; more fertile crosses tended to be grouped while less fertile ones were distributed over a wide range. The titration procedure usually detected all except the most infertile crosses, because the ten step sperm dilution series usually started with high enough sperm concentration to achieve at least 4% fertilization in the first three wells. The most fertile crosses were more often lost from the analysis than infertile ones, because the dilution series had not been long enough, and more than 90% fertilization occurred even at the greatest sperm dilution. Thus the ranges observed were probably less than actually occurred and the observed mean values of sperm/fertilization were probably too high because of limitation due to the method. The proportion of crosses that were "unusable" due to too high sperm concentration varied with the species; that is, crosses within the most fertile species were missed more often than those within species that generally required more sperm/fertilization. Thus, the calculated means are probably more accurate for those species with the lowest gamete fertility, than for those species that had more fertile gametes. Variation in sperm fertilizing capacity dominated in this analysis, also, in that unusually high or low values were often associated with a particular male, which tended to be consistent when crossed with various females. Seasonal and geographical effects were not apparent from these results and should be examined more specifically. It would also be of interest to compare crosses between animals from the same area with crosses between animals from different collecting sites.

There were probably species differences in mean gamete fertility. Comparisons were uncertain because of the abnormal distribution of the data but some differences are apparent from the analysis summarized in Table V. It is most probable that *P. indiana* gametes were, on the average, less fertile than those from all other species, when the data were compared in three different ways ($P < 0.001$ in all cases). On the same basis, gametes from *E. mathaei* were less fertile than those from *H. mammillatus* or *T. gratilla.* Log conversion and adjustment of the data by omitting some of the least fertile crosses (see Table V) agree to suggest that *E. mathaei* gametes were significantly different from those of *H. mammillatus,* or *T. gratilla.* Finally, the data adjusted by omitting some of the least fertile crosses suggest that gametes from *E. oblonga* might have been less fertile than those from *C. atratus,* and *T. gratilla* gametes might have been different from those of *C. atratus* and *H. mammillatus. H. mammillatus* gametes were probably not different from those of *C. atratus* and gametes from the two species of *Echinometra* were also probably of about the same fertility.

Some species that were alike in gamete fertility were closely related taxonomically. *C. atratus* and *H. mammillatus* are both members of the family Echinometridae of the order Echinoida (Fell, 1966). They are further alike in that both have large, flattened primary spines on the oral surface and around the periphery of the flattened test. The primary spines are limited to those areas on *C. atratus,* but in *H. mammillatus,* occur also on the aboral surface where they are large club-shaped structures with a more or less triangular cross section. *C. atratus* lives limpet-fashion on rocks high in the surf zone while *H. mammillatus* lives in crevices in the rocks, either in the surf zone or subtidally. The primary spines of both species are used to lock the animal in place on the rocks. In both species the secondary spines are short, with flat ends, and form a mosaic-like covering on the aboral surface.

The two species of *Echinometra,* are morphologically almost alike and frequently occur together (Kelso, 1970). *E. oblonga* is dark colored and most often found in the surf zone, or just subtidally, while *E. mathaei* is light colored and is usually subtidal. The two forms have been classified as subspecies of *mathaei* (Mortensen, 1943), but are probably distinct species because it was difficult to cross fertilize their gametes (only about half of the attempted crosses had more than 4% activation, and of those, most had less than 10%, see Table VI). Gametes were obtainable from both for the same season and presumably occurred together in nature.

Tripneustes and *Pseudoboletia* are also close taxonomically (Table I), but gametes from *T. gratilla* were, on the average, at least 10 or perhaps 50 times more fertile than those from *P. indiana.* Individuals of both species were found in the same subtidal locations, although *T. gratilla* were by far the most abundant. Both kinds were of about the same size, released about the same numbers of gametes following KCl injection (100×10^6 eggs, or 200×10^9 sperm from average individuals), yielded gametes the year around and were observed to spontaneously release gametes at the same time and place. Virtually no activation occurrd in the 25 attempted crosses between the two species (Table VI).

Variation in gamete fertility is of considerable importance to the study of reproduction. Such variation not only influences results of experiments but also can be of value as a way of evaluating the mechanisms of gamete interaction. Comparisons between gametes of high or low fertility, say from extreme cases within a species or between species like *T. gratilla* and *P. indiana,* could, for example, be used to elucidate the factors that characterize potentially successful gamete interactions.

SUMMARY

(1) A technique was developed to determine the number of sperm present for each fertilization, under standardized conditions. Gametes from six species of urchins were examined.

(2) The reliability of the method was examined by analyzing 16 repeated crosses between the same set of gametes. Variability was evaluated by crossing gametes from four males and four females in the 16 possible combinations simultaneously. The results from many such crosses were pooled for statistical analysis.

(3) A wide range of values was obtained for each species and the data were not normally distributed when all values were considered.

(4) There were probably differences between the average fertility of gametes from several of the species examined while other species produced gametes of about the same average fertility as those from other taxonomically closely related species.

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IMMUNOLOGICAL STUDIES OF THE SPERM AND SEMINAL FLUID IN THE HORSESHOE CRAB *LIMULUS POLYPHEMUS* L. (MEROSTOMATA)

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The major physiological events of fertilization are (1) sperm-egg interactions prior to attachment, (2) interactions after attachment of the gametes is established, (3) activation of the egg, and (4) union of the two pronuclei to form the zygote nucleus (Monroy, 1965). Important aspects of the interactions include the sperm acrosomal reaction, lysins released from the acrosomal vesicle, gamete attachment, and fusion of the gamete plasma membranes.

Since a sperm usually reacts only with an egg of the same species, some mechanism of specificity is involved. The fact that the sperm normally reacts with the egg and not with other cells in the same organism makes the reaction even more specific. The macromolecules which are involved may be an integral part of the plasma membrane or adsorbed to the gamete surface from material in the reproductive tract. The specificity suggests that macromolecules of the sperm and egg interact in a manner comparable to enzyme-substrate or antigen-antibody reactions (Metz, 1967). The initial gamete attachment may involve electrostatic binding with divalent cations possibly participating. Upon closer contact there would be more specificity due to short range forces and steric factors (Perlmann, 1959). The purpose of chemical investigations of fertilization is to understand the nature and sequence of these events on a molecular level (Monroy, 1965).

Ouchterlony immunodiffusion techniques and cross absorption-agglutination experiments with a number of crustacean decapods have indicated that there are at least three surface antigens of *Limulus* sperm (Mowbray, Brown, and Metz, 1970). The present study is a further investigation of *Limulus* sperm antigens.

MATERIALS AND METHODS

Specimens of the horseshoe crab *Limulus polyphemus* L. were obtained from Florida Marine Biological Specimen Company, Panama City, Florida and Marine Biological Laboratory Supply Department, Woods Hole, Massachusetts. The animals were kept in "Instant Ocean" aquaria with circulating, aerated artificial sea water at 15° C.

Preparation of antigens

Spawning of gametes from male or female animals was artificially induced by electrical stimulation (Shrank, Shoger, Schechtman and Bishop, 1967). For sperm studies semen from several animals was combined to obtain approximately

3 ml. The semen contained about 9 × 10^9 spermatozoa/ml. Centrifugation at 480 × g at 5° C for 30 min, separated the semen into three layers: (1) sperm cells, (2) seminal particles, and (3) seminal plasma. The seminal particles and seminal plasma were centrifuged again for better separation. The sperm layer was diluted to 10.6 ml with sea water and centrifuged again in order to wash the spermatozoa.

Sonification was used to break the sperm cells into three parts—acrosomal caps, nuclei, and flagella. A 10% sperm suspension was fixed in 2% formaldehyde in sea water in order to prevent cellular disintegration during the sonification process. Another sperm component, the axial rod, was exposed by treating sperm cells with double strength sea water, thus producing the "false acrosome reaction" (Andre, 1963; Shoger and Brown, 1970). Unsuccessful attempts were made to induce the true acrosome reaction.

Soluble sperm antigens were obtained in frozen-thawed extracts made with liquid nitrogen. One ml of concentrated spermatozoa was fixed with 2 ml of Tris buffer (pH 7.4) and was frozen and thawed three times. This treatment formed an extremely viscous mass. Viscosity was reduced by adding two drops of 0.05% deoxyribonuclease (Nutritional Biochemical Corp., 1 crystallized). Frozen-thawed extracts were also made with concentrated seminal particles, diluted with an equal volume of Tris buffer.

In some experiments, frozen-thawed sperm extract was absorbed with whole eggs to remove any sperm antigens which react with the egg surface. One drop of sperm extract was mixed with at least 500 eggs for this absorption. The same results could usually be obtained with about 300 eggs, but with fewer eggs absorption was apparently not complete.

An egg extract was prepared from 1 ml eggs in 4 ml Tris buffer. This suspension was sonified 1 min, frozen-thawed twice at −20° C, and centrifuged at 3020 × g to remove large pieces of cellular debris. A heart extract was also made by crushing, sonifying, and freezing heart tissue (washed extensively) in Tris buffer.

Preparation of antibodies

Antibodies to *Limulus* sperm antigens were produced in rabbits. Spermatozoa mixed with physiological saline and Freund's complete adjuvant (Fisher Scientific Co., Chicago, Illinois) were injected subscapularly. The sera from different rabbits were usually pooled. Both serum and the extracted globulin fraction (150 mg protein/ml) were used from the experiments. The globulin was obtained by adding an equal volume of saturated ammonium sulfate to cold serum. Both serum and globulin were absorbed with whole sperm cells. Absorbed and unabsorbed antibodies were then used in agglutination, immunodiffusion, and immunoelectrophoresis experiments. In all cases in which the globulin fraction and the whole serum were used and compared, the results were similar. Antibodies to *Limulus* eggs and heart tissue were obtained by methods like those described for the sperm.

Agglutination experiments

Agglutination tests (Quinn, 1968) were used to determine the presence of surface antigens on the sperm and seminal particles. One drop of the antiserum or globulin fraction to be tested was added to one drop of a suspension of spermatozoa or seminal particles. The following materials were used as controls: (1) rabbit normal sera, (2) anti-egg sera, (3) anti-heart sera, (4) anti-sperm sera absorbed with whole sperm cells, and (5) sea water. Each type of test was repeated at least three times with the same results.

Immunodiffusion and immunoelectrophoresis analysis

Ouchterlony immunodiffusion tests (Ouchterlony, 1968; Crowle, 1960) and immunoelectrophoresis (Nerenberg, 1966; Cawley, 1969) were used to study the number and distribution of soluble sperm antigens. The optimum antigen and antibody concentrations for immunodiffusion and immunoelectrophoresis tests were determined by varying the concentrations of both reactants. The results reported in Table II are based on at least ten trials. The minimum number of consistent bands are shown.

RESULTS

Agglutination tests

Agglutination experiments were performed on whole spermatozoa, "false reacted" spermatozoa, separated sperm parts, and seminal particles in order to test for surface antigens (Table I). The agglutination capacity of the following agents was tested: (1) anti-sperm sera, (2) anti-sperm sera absorbed with whole spermatozoa, (3) anti-egg sera, (4) anti-heart sera, and (5) normal sera.

Spermatozoa agglutinated upon addition of whole anti-sperm sera (Figs. 1 and 2). The spermatozoa were attached in the following ways: (1) head to head, (2) flagellum to flagellum, and (3) head to flagellum. The head-to-flagellum agglutination was least prevalent. Spermatozoa which had undergone a "false acrosome reaction" agglutinated in the same manner as unreacted spermatozoa. The axial rod was not involved in the attachment between sperm cells.

Anti-sperm sera absorbed with whole spermatozoa, anti-egg sera, anti-heart sera, and normal sera initially had no noticeable effect on sperm suspension. Head-to-head agglutination of spermatozoa did occur in all four sera after approximately 1 hr. Normal sera diluted 1:80 did not cause agglutination within 6 hrs, whereas anti-sperm sera of the same dilution caused agglutination of spermatozoa within 1 hr.

Sonification broke spermatozoa into nuclei, acrosmal caps, and flagella (Fig. 3). Anti-sperm sera caused agglutination primarily between like parts (Fig. 4). To a lesser extent, caps were seen adhering to flagella (Fig. 5). As in the agglutination tests with whole sperm cells, the other types of sera had no visible effect at first, but in approximately 1 hr nonselective agglutination of acrosomal caps and nuclei began to occur.

The seminal particles are all approximately spherical in shape, but they vary in size and refractiveness (Fig. 1). Seminal particles of all sizes were strongly agglutinated by anti-sperm sera (Table I). Agglutination between particles of different sizes shows that these particles have at least one antigen in common, but not all of the seminal particles are necessarily alike. Anti-sperm sera absorbed with sperm cells produced the same effects on the particles as did whole anti-sperm

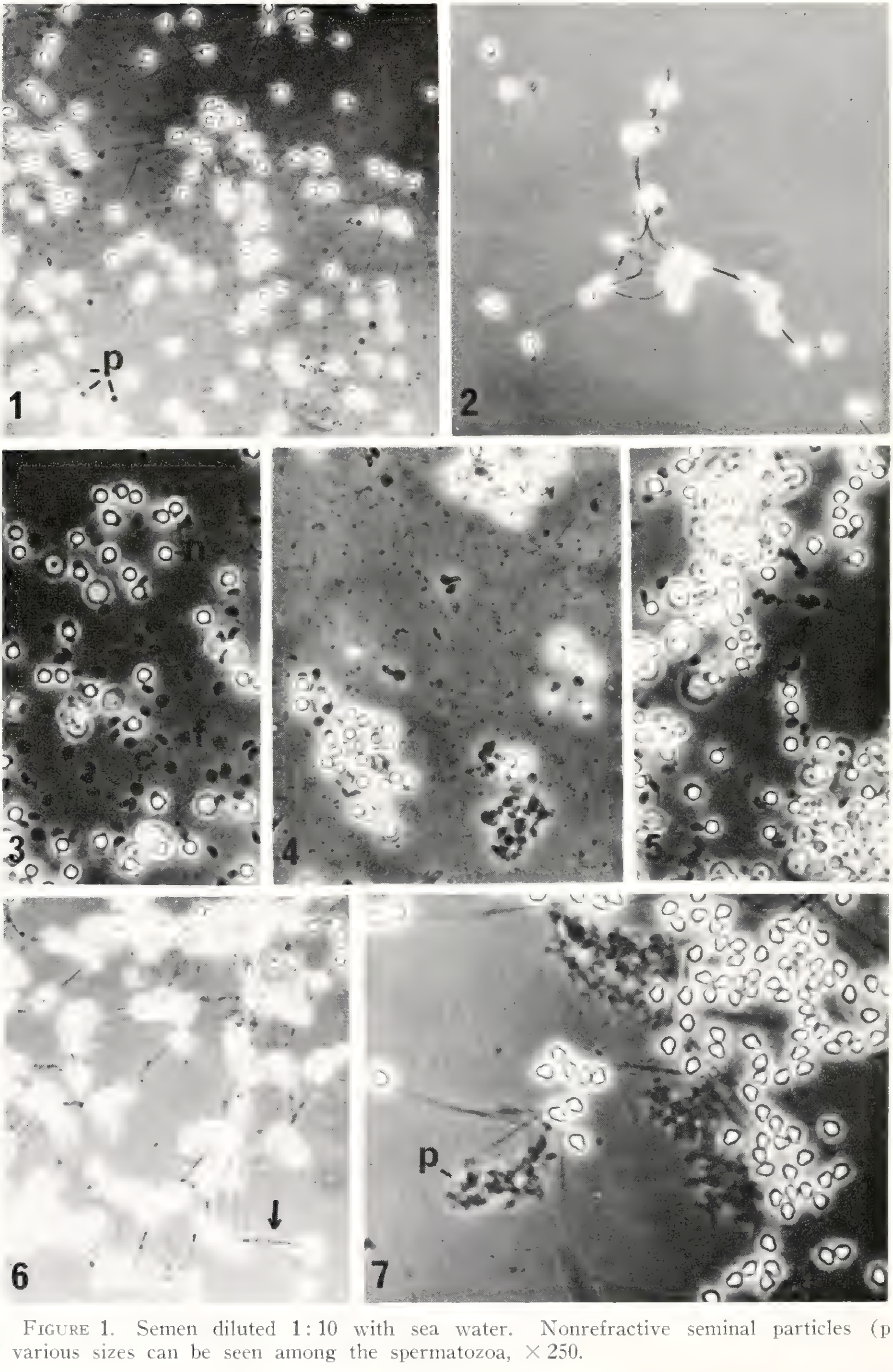

FIGURE 1. Semen diluted 1 : 10 with sea water. Nonrefractive seminal particles (p) of various sizes can be seen among the spermatozoa, × 250.

sera in agglutination tests. When whole semen was used, some seminal particles became attached to the sperm flagella (Fig. 6). This phenomenon did not occur with control sera. When anti-sperm sera absorbed with sperm cells was used with whole semen, the particles agglutinated but did not attach to sperm flagella (Fig. 7).

These agglutination tests using whole sperm, dissociated sperm parts, and seminal particles demonstrated at least three sperm surface antigens and two surface antigens on the seminal particles. One of the surface antigens is shared by the seminal particles and the sperm flagella. The seminal particles have at least one surface antigen which is not present on the sperm surface since the particles are agglutinated by sera absorbed with whole sperm.

TABLE I

Results of agglutination experiments. Agglutination tests were performed with anti-sperm serum. Symbols indicate degree of agglutination of seminal components (sperm, sperm nucleus, etc.) listed horizontally to those listed vertically

	Sperm	Sperm nucleus	Acrosomal cap	Flagellum	Axial rod	Seminal particle
Sperm	**	—	—	—	0	*
Sperm nucleus	—	**	0	0	—	0
Acrosomal cap	—	0	**	*	—	0
Flagellum	–	0	*	**	—	*
Axial rod	0	—	—	—	0	0
Seminal particle	*	0	0	*	0	**

** Strong agglutination.
* Moderate agglutination.
0 No agglutination.
— No test performed.

Immunodiffusion and immunoelectrophoresis analysis

Information about the number and location of sperm antigens and their relationships to antigens of the seminal fluid, the egg, and the heart was provided by immunodiffusion and immunoelectrophoresis tests. Frozen-thawed sperm extracts

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FIGURE 2. Washed spermatozoa agglutinated by anti-sperm serum. Note head to head, head to flagellum, and flagellum to flagellum attachments; sperm dilution: 1:20, × 250.

FIGURE 3. Washed spermatozoa broken into parts by sonification. Note highly refractive nuclei (n), nonrefractive acrosomal caps (c), and flagella (f). Sperm cells fixed with 2% formaldehyde before sonification; sperm dilution: 1:25, × 400.

FIGURE 4. Sperm parts agglutinated by anti-sperm serum. Note segregation of nuceli and acrosomal caps into separate aggregates, × 400.

FIGURE 5. Sperm parts agglutinated by anti-sperm serum. Note attachment of several acrosmal caps to one flagellum, × 400.

FIGURE 6. Whole semen, diluted 1:10, and anti-sperm serum. Note seminal particles attached to the flagella of the agglutinated spermatozoa, × 250.

FIGURE 7. Whole semen and anti-sperm serum absorbed with whole sperm cells. Note that the spermatozoa are not agglutinated, but agglutination of the seminal particles is considerable. Light pressure applied to the slide would cause the sperm to drift apart but not the particles, × 400.

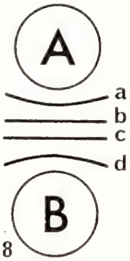

FIGURE 8. Immunodiffusion; (A) anti-sperm serum, (B) frozen-thawed sperm extract from washed spermatozoa; (a), (b), (c), (d) precipitation bands.

produced a minimum of four bands with anti-sperm globulin (Fig. 8; Table II). Immunoelectrophoresis of the frozen-thawed extracts yielded at least seven bands (Fig. 9).

Tests with absorbed anti-sperm sera were made to determine the number of surface and subsurface antigens (Table II). Sperm extracts reacting with absorbed sera produced only two precipitin bands as compared with four to seven bands formed with unabsorbed sera (Figs. 9 and 10a). These experiments revealed that a minimum of five soluble antigens are associated with the sperm surface of *Limulus*. Anti-sperm sera absorbed with "false reacted" sperm cells produced the same result as anti-sera absorbed with normal sperm cells.

Immunodiffusion tests revealed that some of the sperm antigens are related to antigens found in the other seminal components (Table II). There are at least three soluble antigens associated with the seminal particles in *Limulus*. Two of these antigens are identical to two soluble subsurface sperm antigens represented by bands "a" and "b" (Figs. 8 and 10a). Antigen "b" is also found in the seminal plasma. Another antigen which is present in the seminal plasma and seminal particles is apparently somewhat related to sperm subsurface antigen "a" (band "a," Fig. 8).

TABLE II

Results of immunodiffusion experiments. Anti-sperm sperm was used to test for the presence of sperm antigens in the extracts and seminal plasma. Sperm surface antigens were distinguished from subsurface antigens by reacting sperm extract with serum absorbed with whole spermatozoa. Absorption of sperm extract with whole eggs was used to test for the presence of sperm antigens which react with the egg surface. This table summarizes the results shown in Figure 10

Antigens†	Sperm extract	Sperm surface	Particle extract	Seminal plasma	Egg extract	Sperm extract absorbed with eggs	Heart extract
a	*		*		*	*	*
b	*		*	*	*	*	*
c	*	*					
d	*	*					
Related to a††			*	*			

* Antigen present.

† Antigens a, b, c, and d are demonstrated by bands in Fig. 8.

†† The band for this antigen, show in Fig. 10a, fuses with band "a" but is not identical to it.

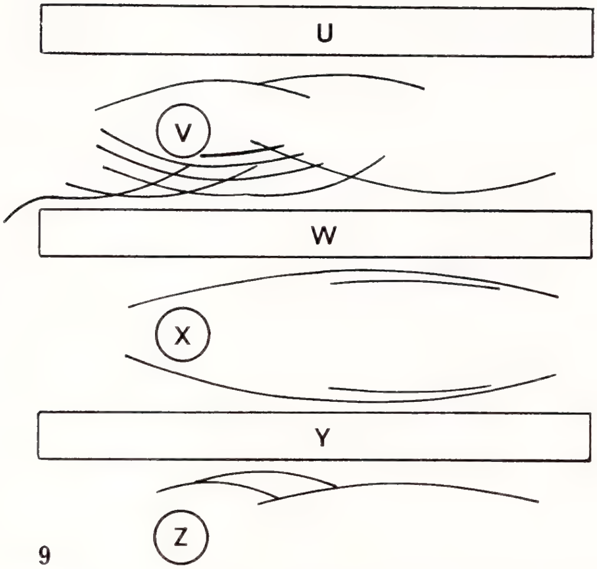

FIGURE 9. Immunoelectrophoresis; (U) anti-sperm serum absorbed with whole sperm cells, (V) frozen-thawed sperm extract from washed spermatozoa, (W) anti-sperm serum, (X) seminal plasma, (Y) anti-sperm serum, (Z) frozen-thawed seminal particles extract.

The sperm cells also have antigens in common with *Limulus* eggs and heart tissue (Figs. 10b and 10c; Table II). Sperm subsurface antigens "a" and "b" are found in both egg and heart extracts.

Frozen-thawed sperm extracts absorbed with whole eggs formed two precipitin bands with anti-sperm sera (Fig. 10b). These two bands correspond to bands "a" and "b" obtained with whole sperm extracts (Table II).

DISCUSSION

Soluble sperm antigens have been studied in a variety of species by means of immunodiffusion techniques. The *Limulus* sperm has at least seven antigens,

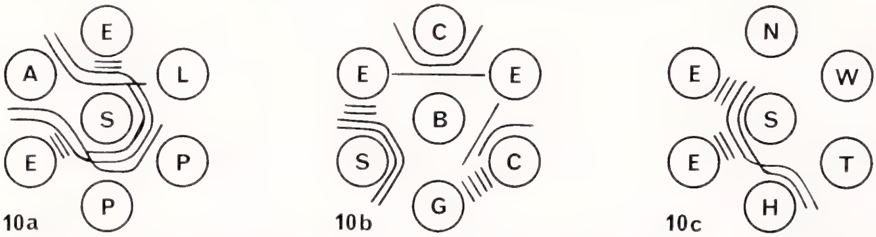

FIGURE 10. Immunodiffusion; (A) anti-sperm serum absorbed with whole sperm cells, (B) frozen-thawed sperm extract absorbed with whole eggs, (C) anti-egg serum, (E) frozen-thawed sperm extract, (G) frozen-thawed egg extract, (H) frozen-thawed heart extract, (L) seminal plasma, (N) normal serum, (P) frozen-thawed seminal particles extract, (S) anti-sperm serum, (T) anti-heart serum, (W) sea water.

whereas four antigens have been identified on the sea urchin sperm (Metz, 1967). Studies on mammals have revealed different numbers of antigens associated with the spermatozoa: (1) four in the guinea pig (Katsh and Katsh, 1961), (2) seven in the bull (Hunter and Hafs, 1964), (3) ten in the Chinese hamster (Piko and Tyler, 1962), and (4) fourteen in the rabbit (Hunter, 1969). The number of sperm antigens demonstrated for *Limulus* is within the range of numbers reported for other organisms in different phyla.

Particular interest is directed toward sperm surface antigens since the initial interactions of the gametes in fertilization must involve macromolecules on the surface of the sperm and egg. Immunoelectrophoresis revealed five sperm surface antigens in the case of *Limulus*, and agglutination tests showed that the antigens are unevenly distributed on the surface. At least one antigen is apparently unique to the surface of the nuclear part of the sperm. The agglutination of acrosmal caps to flagella was probably due to identical or very similar antigens on these sperm parts. Thus the distribution of surface antigens on the *Limulus* sperm may resemble the situation found in the sea urchin sperm (Metz, 1967).

Possible functions which sperm surface components play in fertilization have been summarized by Piko (1969). They are as follows: (1) prevention of sperm agglutination, (2) regulation of membrane permeability, (3) prevention of an early acrosome reaction by covering receptor sites on the plasma membrane, and (4) prevention of an early uptake of the sperm by phagocytes. The antigenic components of the *Limulus* sperm may be involved in similar functions.

Immunological studies have shown that some mammalian spermatozoa become coated with materials from the seminal fluid (Hunter and Hafs, 1964; Weil and Rodenburg, 1962; Hunter, 1969). These findings indicate the importance of studying antigens in the seminal fluid and determining their relationship to the sperm. The present study on *Limulus* indicates that there are two soluble subsurface sperm antigens which are also associated with the seminal particles. One or both of these antigens could be involved in the agglutination of the seminal particles by sera absorbed with whole sperm. The antigen which is found on the surface of the sperm flagellum and on the seminal particles may be insoluble and thus not subject to detection by immunodiffusion tests.

The distribution of antigens in *Limulus* semen is comparable to that in some mammals. For example, some of the antigens of the bovine sperm are also found in the seminal plasma (Hunter and Hafs, 1964). The same situation exists in the Chinese hamster (Piko and Tyler, 1962). Seminal particles possessing some antigens in common with the sperm have been found in the bull (Lindahl and Brattsand, 1962) and the rabbit (Metz, Hinsch, and Anika, 1968). The latter studies showed that the rabbit seminal particles have antigens which are also present in the seminal plasma, and at least one particle antigen is shared by the sperm surface. These results resemble those obtained for the *Limulus* system.

Ouchterlony experiments involving cross reactions between egg or sperm extracts and anti-sera show antigenic relationships between the gametes (Table II). The results suggest that the sperm and egg have two antigens in common. Since these antigens are subsurface ones, at least in the case of the sperm, they are probably not directly involved in the initial sperm-egg interactions. The same two antigens are also found in extracts of *Limulus* heart tissue, so they may be associated with sperm cell structures which do not participate directly in fertilization. A comparative study should be pursued to determine whether these antigens are specific for *Limulus.*

One comparative study dealing with the sperm surface antigens of five crustacean decapods and *Limulus* has been performed (Mowbray, Brown, and Metz, 1970). Cross-absorption-agglutination tests were used to study the species specificity of the sperm surface antigens. This work revealed that at least two sperm surface antigens are not entirely unique to *Limulus.* The *Limulus* sperm shares one surface antigen with the sperm of *Pagurus pollicaris* and another surface antigen with the sperm of *Cancer irroratus.* Since fertilization reactions are usually very specific ones, unique *Limulus* sperm antigens should be distinguished from those which are also found in other types of *Limulus* cells or other species.

The experiments using sperm extracts absorbed with whole eggs suggest that the two surface antigens represented by bands "c" and "d" on Ouchterlony plates react with the egg surface (Fig. 10b). A fertilizin-antifertilizin type of system may be present. Further investigations may reveal that these antigens are involved in the acrosome reaction and initial gamete attachment.

Further immunological studies using isolated sperm parts can be performed for more specific localization of sperm antigens. Such studies combined with staining techniques and enzyme analysis can provide information about the chemical nature of the antigens. The purpose is aimed at the eventual elucidation of biochemical reactions involved in fertilization in *Limulus.*

SUMMARY

Immunological methods were used to investigate *Limulus* sperm antigens. Immunoelectrophoresis experiments revealed seven soluble sperm antigens, and absorption techniques demonstrated that five of these antigens are located on the sperm surface.

Agglutination tests showed that surfaces of the sperm nucleus, acrosomal cap, and flagellum have both different and common antigens. The surface of the acrosomal rod is nonantigenic.

Immunodiffusion experiments revealed that the spermatozoa, seminal particles, and seminal plasma have some antigens in common. The seminal particles are strongly agglutinated by anti-sperm sera and moderate agglutination occurs between particles and sperm flagella.

Cross reactions of anti-sperm sera with *Limulus* egg and heart extracts demonstrated that two sperm subsurface antigens are also present in these other types of cells. Absorption techniques combined with immunodiffusion experiments gave evidence that two sperm surface antigens react with components on the egg surface.

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EVAPORATIVE WATER LOSS BY TARDIGRADES UNDER CONTROLLED RELATIVE HUMIDITIES

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Certain soil-dwelling animals such as rotifers, tardigrades, and nematodes, possess the unique ability, under drought conditions, to be divorced from virtually all their body water. Yet they are not killed. When they come in contact with water the animals swell and resume active life, often within minutes. This phenomenon, which has fascinated biologists since its discovery by Leeuwenhoek, is called cryptobiosis (see Keilin, 1959; Crowe, 1971; Crowe and Cooper, 1971 for recent reviews).

Tardigrades which survive desiccation must be allowed to dry very slowly ("très lentement," according to Broca, 1860, page 1) but exactly what "very slowly" means in quantitative terms or of what significance the slow drying might be to the animal has been only a source of conjectiure.

Davis (1873) believed that slow drying of rotifers enabled the animals to secrete a gelatinous sheath which protected them from desiccation. Jacobs (1909) and Hickernell (1917) disproved this notion by chemical tests for water and direct histological examination, which revealed no such sheath. Others, notably Pouchet (1859a, 1859b), observed that tardigrades or rotifers dried on a clean slide were killed, while those dried in a little sand survived. They believed that sufficient water remained between the sand grains to maintain the animals in a state of partial hydration. Doyère (1842) on the other hand, insisted that drying on a clean slide was not necessarily injurious. Close examination of Doyère's techniques shows, however, that he dried his animals slowly, in a humid atmosphere, while Pouchet took no such precautions. Clearly, the humidity of the surrounding air, and presumably the consequent rate of evaporative water loss, are important to the tardigrade that is to become cryptobiotic.

The focus of this investigation is on the rate of evaporative water loss from tardigrades kept under controlled relative humidities. The aim of the study is to provide a basis for investigations on the mechanism of the induction of cryptobiosis.

Experiments and Observations

Evaporative water loss

The rate of evaporative water loss from tardigrades was investigated by recording weight loss from animals kept at known relative humidities. Specimens of *Macrobiotus areolatus* Murray, 1907, which had been starved for 24 hours immediately before the experiment, were washed in distilled water, picked up individually with an Irwin loop, and blotted with filter paper to remove adhering water. The blotted animals were transferred with an Irwin loop (which was silicone-coated to prevent removal of water from the test droplet) to a precisely measured 5 or 10 μl drop of distilled water on a 5 mm weighing pan. The weighing pan was suspended from the pivot arm of a Cahn RG electrobalance calibrated to ± 0.54 μg. The balance was coupled to a Sargent chart recorder. Relative humidity of the air surrounding the pan was controlled by sulfuric acid solutions of known density (and therefore of known vapor pressure; *cf.* Edney, 1966). The flask containing the sulfuric acid solution was provided with a magnetic stirring bar to insure fast equilibration between the solution and air. The entire apparatus was housed in a constant temperature chamber at 25° C.

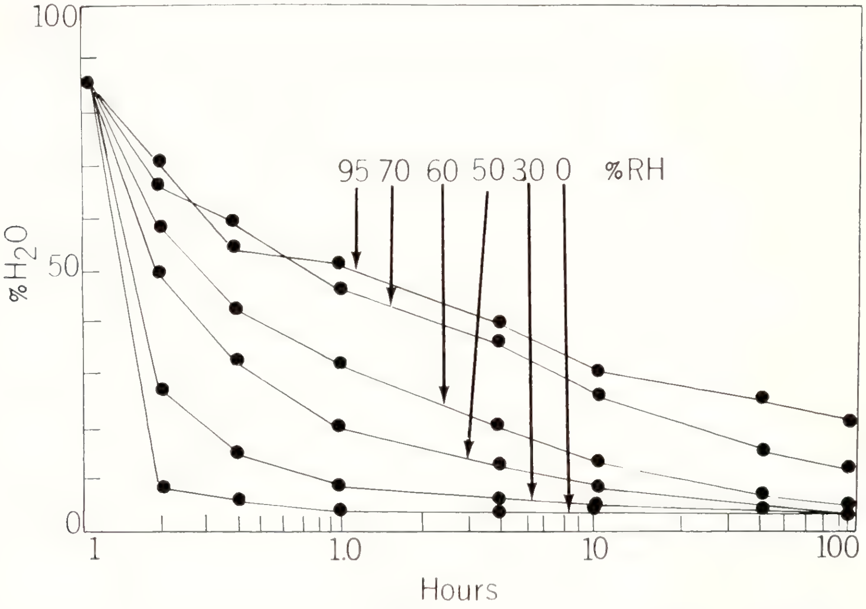


FIGURE 1. Water contents of tardigrades kept at various relative humidities.

The results of these experiments are presented in Figures 1 and 2. Calculation of the per cent water contents was based on the assumption that animals held at 180° C for 3 hours contained no water. Calculations were made according to the equation:

$$\% \ H_2O \text{ content} = \frac{W_t - W_d}{W_t} \times 100,$$

where W_t = weight at time t and W_d = dry weight. The weight of an average, fully hydrated animal was about 98 μg, of which about 12 μg was dry matter and 86 μg was water. All determinations were carried out on groups of about 25 animals.

Over the range of about 70–95% relative humidity (RH) different groups of animals which were active at the beginning of the experiment showed similar rates of evaporative water loss. During the first hour under these conditions active animals were reduced from their original water contents of 80–90% to about

50–60% (Figs. 1 and 2). Active animals were subsequently reduced to an equilibrium water content of 10–25% over a period of about 100 hours.

At relative humidities below 60%, the rate of evaporative water loss by animals which were active at the beginning of the experiment was much greater than at higher humidities (Fig. 1). At 0% RH, for example, the animals were reduced to 2–3% water content in about 15 minutes. Active animals dried under these conditions were killed. Even though such animals could not survive direct exposure to dry air, those which were reduced previously to about 20% water content at 80% RH did survive exposure to dry air. When such animals were placed in dry air, their water content was reduced to 2–3% without killing them.

The differences in the rates of water loss from animals which were active at the beginning of the experiment and animals which were dead or anesthetized animals were highly significant ($P = 0.01$). Animals which were active at the beginning of the experiment retained a significant proportion of their body water for extended periods, while animals which were killed with 2% formalin or 0.1 M KCN or anesthetized with CO_2 were reduced to equilibrium water content within one hour. Active animals lost water at a rate approximately 0.3 times that of anesthetized animals during the first hour under dehydrating conditions. Anesthetized animals not dried regained activity within a few minutes when they were returned to oxygenated water, but the dried animals were killed.

Permeability coefficients (K) were calculated for four arbitrarily selected time periods (Figure 2), according to the equation of Lucké and McCutcheon (1932):

$$K = \frac{dV}{dt(P_1 - P_2)A},$$

where dV = volume of water lost (liquid phase),
dt = time change,
$P_1 - P_2$ = net water potential, and
A = surface area.

A 500 μ long animal with surface area of 2×10^{-4} cm^2 was assumed. Hemolymph osmotic pressure (OP) needed to make these calculations was measured by the indirect freezing point method of Gross (1954). OP showed a range of 2.20–2.87 osmoles/l, with a mean of 2.48 osmoles/l.

During the first stages of dehydration the K's were similar over the entire humidity range, but the K for anesthetized animals was consistently higher than that for animals active at the beginning of the experiment. The K for anesthetized animals dried at 80% RH was nearly 3 times that of active animals dried at the same humidity. In the latter stages of dehydration, the K of animals dried at RH > 70% decreased as much as 100-fold (Fig. 2).

Morphology and cuticular permeability

Active animals (Fig. 3) contract when dried at high humidities and form so-called "tuns" (Fig. 4). Anesthetized animals, on the other hand, simply flatten (Fig. 6) or crumple (Fig. 5).

Crowe, Newell and Thomson (1971a) reported that the cuticular folds (Figure 4), which are flexion points in the cuticle, are about 1/20 the thickness of the rest of the cuticle. This observation suggests that these thin areas may be more highly permeable than the rest of the cuticle. When the animal contracts into a tun (Fig. 4), the thin cuticle areas are drawn into the body wall and removed from contact with the air. If such areas were indeed more permeable than the rest of the cuticle, formation of the tun could then, in part, account for reduced evaporative water loss by tuns compared with animals which were not allowed to form tuns.

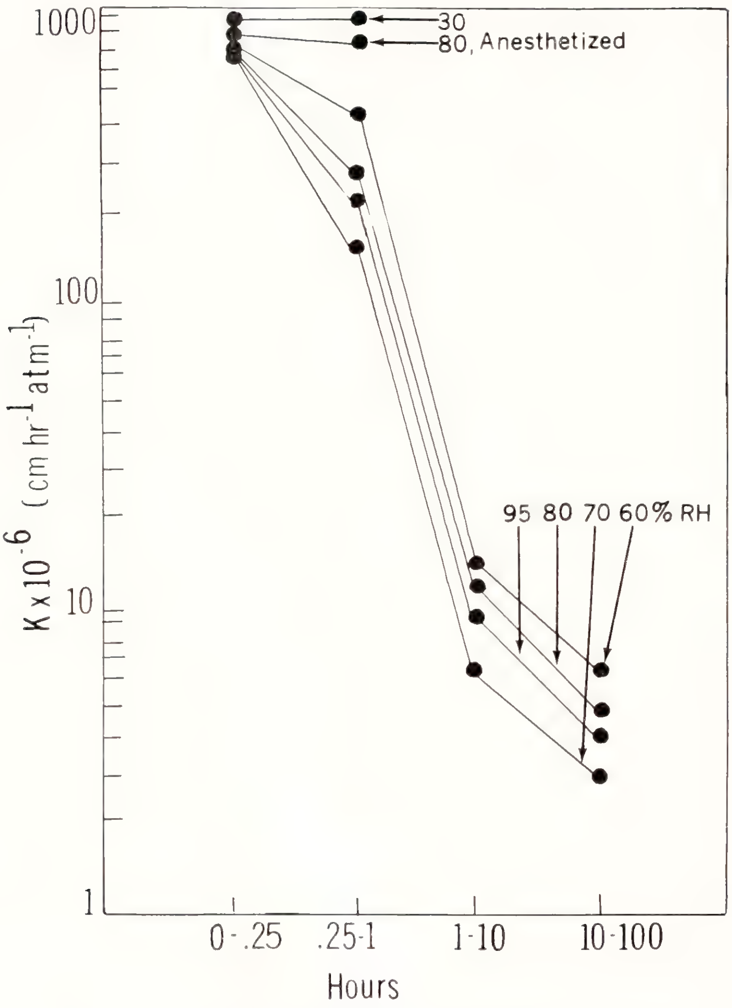
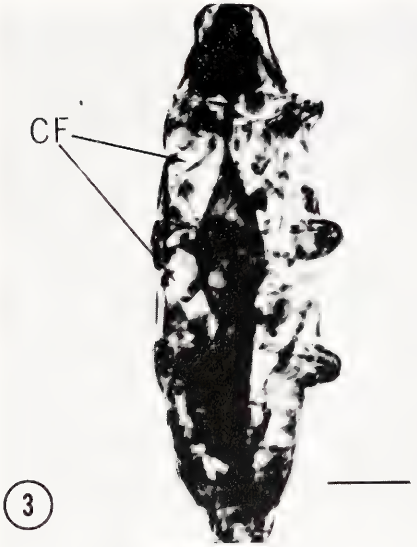
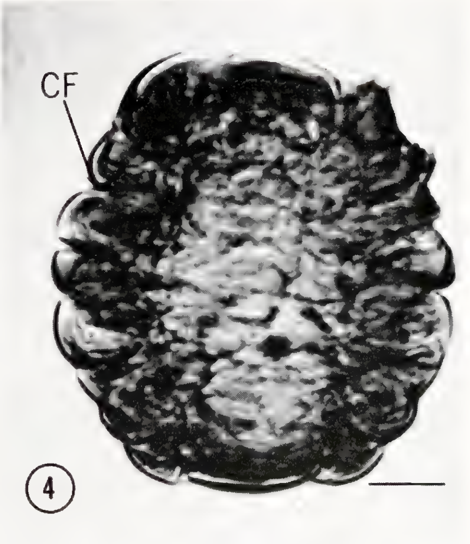
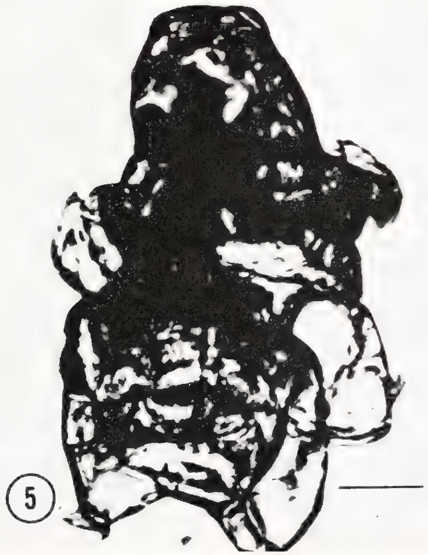


Figure 2. Permeability coefficients of tardigrades dried at various relative humidities. For explanation see text.

Relative permeabilities of the thick and thin areas of the cuticle were studied through the use of dyes. Active animals were immersed in 0.5% crystal violet, periodically transferred to distilled water, and examined with the light microscope. The dye penetrated the thin portions of the cuticle within a few seconds,







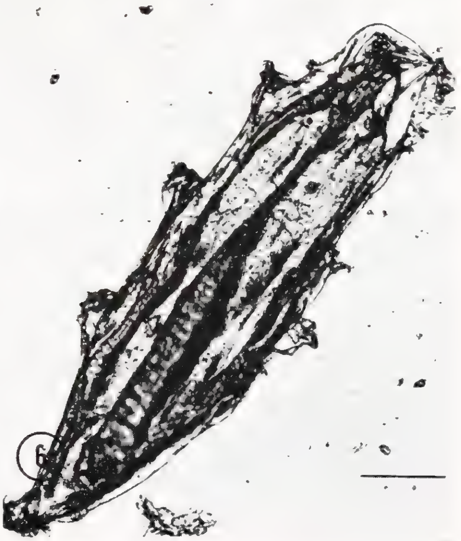
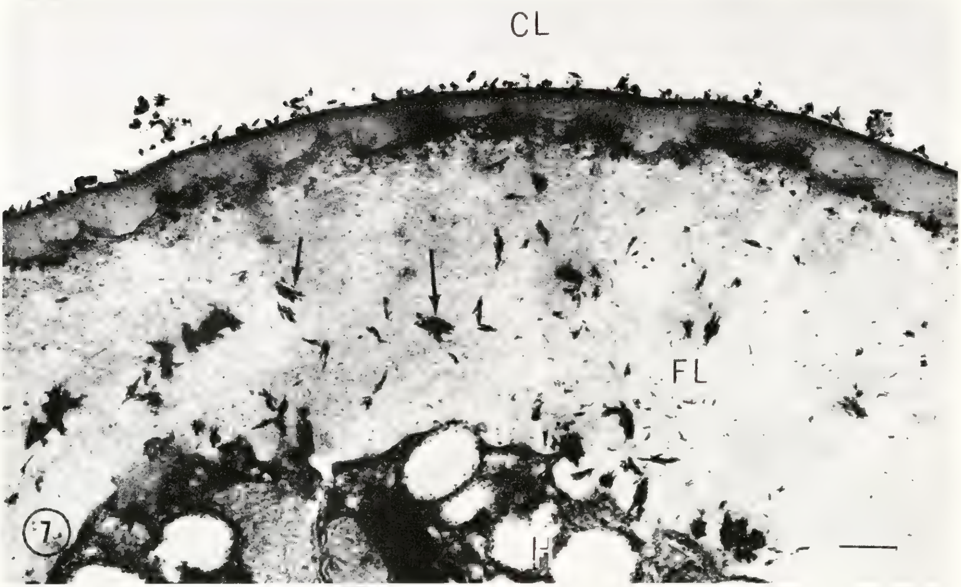


FIGURE 3. Photomicrograph of an active tardigrade; CF = cuticular folds; marker = 50 μ.

FIGURE 4. A tun of *M. areolatus,* obtained by drying an active animal at high humidity; CF = cuticular folds; marker = 50 μ.

FIGURE 5. A specimen of *M. areolatus* that was dried at low humidity; marker = 50 μ.

FIGURE 6. A specimen of *M. areolatus* that was anesthetized before it was dried; marker = 50 μ.



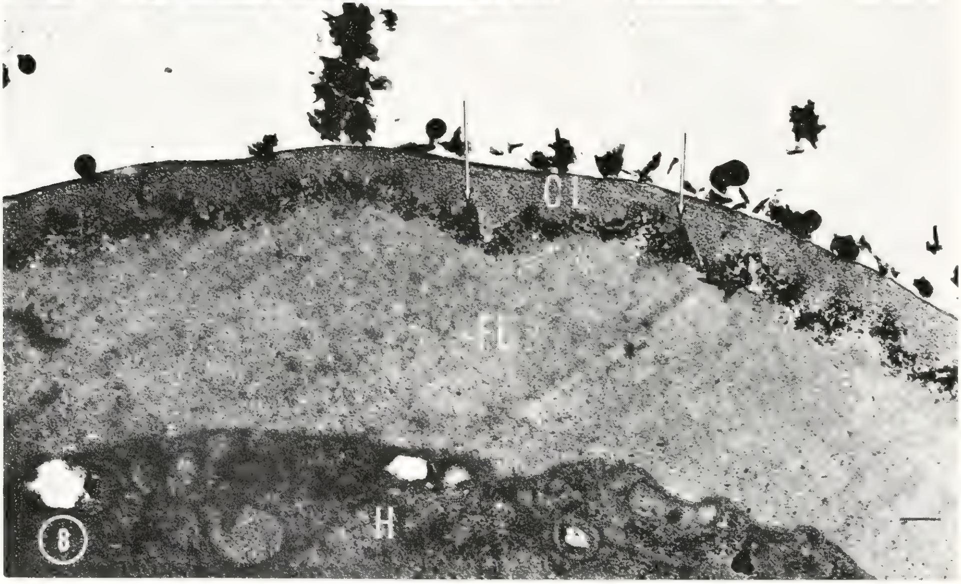


FIGURE 7. Electron micrograph showing the penetration of the cuticle of *M. areolatus* in the active state by lead ions. Note the distribution of lead (arrows) throughout the cuticle and hypodermis; CL = cortical layer; FL = fibrous layer; H = hypodermis; marker = 1 μ.

FIGURE 8. Electron micrograph showing the penetration of the cuticle of *M. areolatus* in the cryptobiotic state by lead ions. Note the accumulation of lead (arrows) at the outer boundary of the fibrous layer; marker = 1 μ.

but it penetrated the thicker portions extremely slowly. Crystals were seen in the hypodermis underlying the thick cuticle only after prolonged exposures of several hours. Alternatively, the animals were vitally stained for 24 hours in 0.1% neutral red, a stain which changes to yellow at alkaline pH. Animals so stained were placed in water at pH 8.5. The color change was seen most intensely at the mouth and anus and along the cuticular folds. It appears, then, that the cuticular folds are areas of high permeability as their morphology suggests.

Sequestration of the high permeability areas of the cuticle by tuns can account for the differences between rates of evaporative water loss by active and anesthetized animals, but it clearly cannot account for the 100-fold decrease in PC seen in the terminal stages of dehydration. This diminution in rate of water loss suggests that the entire cuticle decreases in permeability as it dries. This possibility was investigated by comparing rates of water entry into active tradigrades and tuns. The animals were stained with neutral red as previously described. Active animals placed in water at pH 8.5 showed a color reaction at the mouth, anus, and cuticular folds most strongly, but also showed a weak reaction along the thick cuticle, indicating that at least some water penetrates through this cuticle. Tuns placed in water at pH 8.5, on the other hand, showed a strong reaction at the mouth and anus, but no reaction in other portions of the cuticle for several seconds. These results, which suggest that the cuticle does indeed decrease in permeability when dried, were confirmed in the following way. Active animals and tuns were placed in 2% aqueous $PbNO_3$ for 10 sec and immediately transferred to 4% paraformaldehyde in 0.1 m phosphate buffer, the phosphate ions trapping Pb^{++} as $PbPO_4$. The animals were then processed for electron microscopy as previously described (Crowe *et al.*, 1971a). The results of this experiment show that lead penetrates the cuticle readily; $PbPO_4$ crystals were seen scattered through the cortical and fibrous layers of the cuticle of active animals (Fig. 7). In tuns, however, penetration is much reduced; $PbPO_4$ crystals were seen only in the cortical layer, never in the fibrous layer (Fig. 8).

DISCUSSION

These studies have provided a more precise analysis of the effects of relative humidity on survival and desiccation rate of tardigrades in air than was previously possible. The highest number of survivals was obtained when the animals were dried at relative humidities greater than 70%. This is in some way related to the fact that at high humidities the tardigrades retained significant proportions of their body water for extended periods of time—up to about 100 hours (Figs. 1 and 2). At lower relative humidities they lost water at a much greater rate, reaching equilibrium within 1 hour (Fig. 1). At high humidities the animals contract into a tun (Fig. 4), while anesthetized animals or animals dried at lower humidities become irregularly crumpled (Fig. 5) or simply flattened (Fig. 6). The difference in the rate of water loss by tuns and non-tuns is probably due to the fact that in tuns, high permeability areas of the cuticle are removed from contact with the air.

The rate of evaporative water loss continues to fall for some time, even after the animals have contracted into tuns; during the first hour at 80% RH tuns lose water at a rate of about 34 mgm cm^{-2} hr^{-1}, 9 mgm cm^{-2} hr^{-1} during the 1–10 hour period, and 0.7 mgm cm^{-2} hr^{-1} during the 10–100 hour period (calculated from Fig. 1 and 2). These rates are similar to those shown by aquatic insects in air. Holdgate (1956) found that evaporative water loss from such insects was 0.3–11.5 mgm cm^{-2} hr^{-1}. The decrease in rate of evaporative water loss appears, from the evidence presented above, to be due to a decrease with desiccation of the permeability of the cuticle to water. Such decreases are not unknown. For example, Edney (1951) Auzou (1953), and Bursell (1955) found that when isopods are exposed to dehydrating conditions, the rate of evaporative water loss falls for some time before it reaches steady state. A similar phenomenon has been reported for certain aquatic insects by Bursell (1955) and Holdgate (1956). Conversely, most terrestrial insects achieve constant rates of evaporative water loss rather quickly (Beament, 1958, 1961).

It is significant that the arthropods that exhibit decreases in cuticular permeability under dehydrating conditions lack extra-cuticular lipids, but possess endocuticles that are rich in lipo-proteins (see Edney, 1957 for references). The cuticle of *M. areolatus* also lacks extra-cuticular lipids, but the fibrous layer of the cuticle (which appears to be the major permeability barrier) is rich in lipoproteins (Crowe *et al.,* 1971). Bursell (1955) suggested that dehydration of the cuticles of isopods brings epicuticular lipids in close proximity to each other, which results in a permeability decrease.

If the cuticle is really important to the tardigrade in controlling its rate of desiccation, an obvious difficulty is the molt. Crowe, Newell and Thomson (1971b) have shown, however, that in *M. areolatus* the old cuticle is maintained intact until the new cuticle is completed. It appears that the old cuticle retains its function until the new one is completed.

It seems now that slow evaporative water loss is necessary for the survival of desiccation by tardigrades. Similar observations have been made by Ellenby (1968). He found that nematodes undergoing desiccation congregate into groups, forming a "nematode wool." Those individuals on the inside of the group dry more slowly and survive desiccation better than those on the outside. Ellenby also presented some evidence that the cuticle of nematodes undergoing desiccation decreases in permeability.

The most interesting questions yet to be answered center around the necessity for slow evaporative water loss by animals entering a cryptobiotic state. That water loss must be slow implies that the animals must prepare in some way for extensive desiccation. I have presented hypotheses elsewhere concerning the nature of the preparative process (Crowe, 1971; Crowe and Cooper, 1971).

I gratefully acknowledge the advice and encouragement of E. B. Edney, I. M. Newell and W. W. Thomson during these studies. Supported in part by grants ES00084 from the National Institute of Environmental Health Sciences and D-686 from the University of California.

SUMMARY

The effects of relative humidity on the survival of desiccation by the tardigrade, *Macrobiotus areolatus*, were investigated. The most survivals were obtained when

the animals were dried at relative humidities greater than 70% at 20° C. At these high humidities the animals form tuns, while at lower humidities they become flattened or crumpled. Anesthetized animals do not form tuns at any humidity.

The rate of evaporative water loss from tuns in air was investigated by recording weight loss from animals kept at known relative humidities. Tuns formed by active animals lose water during the early stages of dehydration at a rate approximately 0.3 times the rate of anesthetized animals. Anesthetized animals equilibrate with the surrounding air within one hour, while tuns require more than 100 hours to equilibrate. At the end of 100 hours, the water content of tuns at 80% RH is 10–25%. During dehydration the permeability coefficient of tuns decreases a hundredfold (from $2.0–5.0 \times 10^{-4}$ cm hr^{-1} atm^{-1} to $1.6–6.0 \times 10^{-6}$ cm hr^{-1} atm^{-1}). The tardigrades can be reduced to 2–3% water content in dry air without killing them, if they are first dehydrated to about 20% water content at 70–95% RH.

The cuticle was investigated as the most likely site of control of evaporative water loss. Studies with dye and heavy metal tracers demonstrated that during tun formation high permeability areas of the cuticle are removed from contact with the air. After extensive dehydration the entire cuticle becomes less permeable to water, possibly due to a lipid phase change.

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REPRODUCTIVE AND MATERNAL BEHAVIOR OF THE MANTIS SHRIMP *GONODACTYLUS BREDINI* MANNING (CRUSTACEA: STOMATOPODA)

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The mantis shrimp *Gonodactylus bredini* is an active predatory marine crustacean distributed in tropical and sub-tropical waters of the Western Atlantic (Manning, 1969). It lives in cavities in rocks and rubble in littoral and sub-littoral zones and defends these cavities vigorously. This defense involves elaborate behavior patterns including spreading of the raptorial meri in a posture which appears to serve as threat and use of the raptoral appendages to strike an opponent. Details of this aggressive behavior have been analyzed utilizing extensive records from homosexual interactions (Dingle, 1969a; Dingle and Caldwell, 1969). This behavior is apparently characteristic of the family Gonodactylidae since it occurs also in *G. oerstedi* (Caldwell, unpublished), *G. spinulosus* (Dingle, 1971), *Haptosquilla (Gonodactylus) glyptocercus* (Serène, 1954) and in several Pacific and Indian Ocean species (Caldwell and Dingle, unpublished).

Species of *Gonodactylus*, along with those from other genera and families of stomatopod, also exhibit an elaborate maternal behavior (Giesbrecht, 1910; Verrill, 1923; Serène, 1954). The female lays her eggs in a compact mass which she constantly kneads with her maxillipeds; she remains with eggs and newly hatched larvae until the latter mature to the planktonic stage and leave the burrow or cavity (Dingle, 1969b). The only description of stomatopod courtship in the literature is that of Serène (1954) who describes it for *Haptosquilla glyptocercus*.

The initiation of courtship behavior in *Gonodactylus* must involve highly specific communication between potential mates to insure that pre-copulatory rather than aggressive interactions take place. Indeed in many heterosexual encounters the interaction is an aggressive one (Dingle and Caldwell, 1969). There must therefore be some exchange of signals leading to courtship rather than to aggression. Elaborate signaling during courtship is of course well-known in semi-terrestrial crabs (Crane, 1966; Salmon and Atsaides, 1968; Schöne, 1968; Wright, 1968), shrimp (Johnson, 1969), and various hermit crabs (Hazlett, 1966, 1968). In view of the widespread interest in crustacean behavior and communication and the complexity of behavior in *G. bredini*, a study of reproduction in this species seemed of interest.

MATERIALS AND METHODS

The species *Gonodactylus bredini* has previously been described as *G. oerstedi* (Verrill, 1923; Hazlett and Winn, 1962; Dingle, 1964), but the two species are now recognized as distinct (Manning, 1969). *G. bredini* is the only one recorded from Bermuda although the two are sympatric and occupy similar habitats throughout the West Indies.

This study was done at the Bermuda Biological Station, St. George's West, Bermuda. Most data were taken during June and July, 1968, with a few observations from the same months in 1966.

The animals were obtained by breaking up pieces of rock collected in shallow water at low tide. They were returned to the laboratory and maintained in sea water either constantly running or changed daily. Some animals were supplied with chambered rocks similar to those they occupied in nature, but most were induced to occupy 50 or 100 ml Erlenmeyer flasks. These flasks were wrapped in black polyethylene in which a flap was cut to permit observation of the interior without disturbing the animal. All animals were fed daily with pieces of various fish and crustaceans. Each individual or pair was housed in its own container; this is necessary because of the aggressive nature of these animals.

All courtship and copulation sequences were observed in an open flat-bottomed bowl, 19 cm in diameter, with a sand covered bottom. Observations on maternal behavior were made on females housed in polyethylene covered Erlenmeyer flasks.

RESULTS

Courtship and copulation

A courtship sequence leading to copulation is initiated when either the male or the female approaches the other individual. If the male approaches first he spreads the raptorial meri two or three times and then may swim over the female; he then usually palpates her body with his antennules although not uncommonly this is omitted. With or without antennular palpation, the male then grasps the female with his maxillipeds most often at the telson. He then works his way up her body until he is holding her by the carapace with maxillipeds 4 and 5 using the hooked dactyls to grasp the rim. While he is holding her he strokes her carapace with the small first maxillipeds and with the third maxillipeds which are similar to 4 and 5 (the 2nd maxillipeds are the raptorial appendages and are not used during clasping). All the while the female remains virtually motionless. If the female makes the initial approach, she does so from the front or side of the male, not from the rear. When she reaches him, she pushes her head and carapace beneath his cephalothorax which usually induces him to grasp her carapace as indicated above. While still grasping the female's carapace, the male curls his abdomen forward while the female turns half over on her back (Fig. 1); he then brings his gonopods up to oppose her genital openings which are paired submedian structures ventrally located on the sixth thoracic segment. The male gonopods are a pair of tubular structures located at the bases of the last pair of walking legs on the last (eighth) thoracic segment. For the next 10 to 50 seconds the pair remains thus with the male firmly clasping the female's carapace behind the eyes and making thrusting movements with his abdomen while the female is completely relaxed. At the completion of copulation, either the male releases and swims slowly away while the female turns back over on to her ventral surface or the female pushes the male off with her telson and struggles free. Immediately after separation the male may strike the female gently with the raptorial appendages; this post-copulatory strike resembles the aggressive strike in form, but is delivered at much slower speed and with very little force. A tap would be a good description for it.

The courtship-copulation sequence takes place only if the female is receptive. If she is unreceptive, she attacks all other individuals, male or female, which are placed with her. In most cases, it is the female which determines subsequent behavior after initial contact; in 20 out of 22 heterosexual aggressive interactions the female attacked first. This attack consisted of a rapid approach to the male followed by a strike delivered with the raptorial appendages (for detailed description see Dingle and Caldwell, 1969). The attack and strike in all but one case took place within the first 15 seconds after the animals were paired; the one exception took approximately 30 seconds. Receptive females were reluctant to attack as indicated by three encounters between pairs of receptive females. Eventually these pairs fought, but only after an interval of 1–3 minutes. The interval before two males began fighting varied from a few seconds to about one minute.

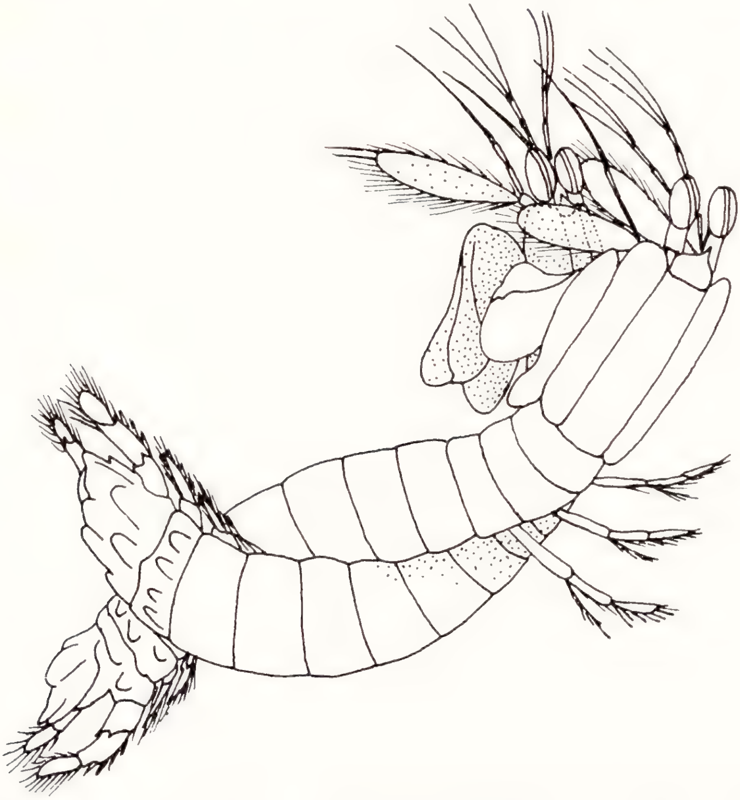

FIGURE 1. Mating in *Gonodactylus bredini* with male above clasping carapace of female below (stippled).

A receptive female can generally be distinguished by the appearance of the ovary and hepatopancreas complex. These organs run the length of the abdomen dorsal to the digestive tube and project into the telson (Balss, 1938). The portions in the telson are visible through the ventral surface. In non-receptive females the visible portions are light in color and have a dendritic appearance. In the receptive female the portions in the telson are extremely dark and are considerably swollen; it has not yet been possible to determine if ovary, hepatopancreas, or both are responsible for the darkened telson, although morphological comparisons following dissection suggest that changes occur in both (M. Reaka, University of California, personal communication). The externally visible difference is illustrated in Figure 2. In 11 out of 12 cases where the ovary-hepatopancreas complex was dark and swollen, copulation took place shortly after the initial encounter with a male. In the other case the female attacked, but the male was behaving abnormally and in fact died the next day. In one case a female copulated when there was no obvious darkening and swelling. In this case, however, the complex had an unusual mottled appearance which was by no means that normal for an unreceptive female. Female receptivity was not connected with the molt as it is in many other crustaceans (Schöne, 1961; Hazlett, 1966).

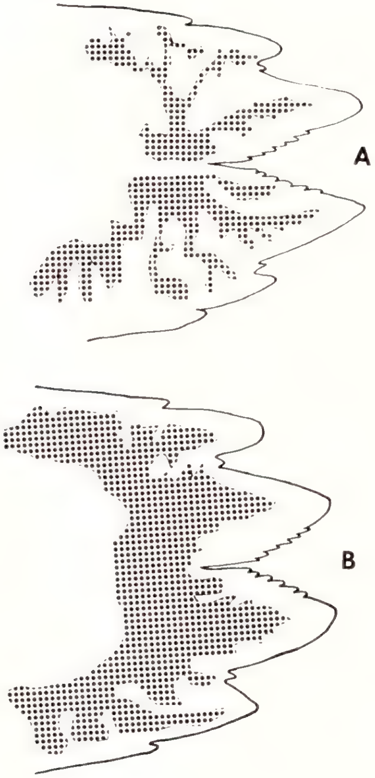

FIGURE 2. Ventral view of telson of (A) non-receptive female and (B) receptive female.

Male receptivity to courtship and copulation is more difficult to determine, and there seem to be no morphological criteria to distinguish receptive males. Most males, however, seem ready to copulate almost continuously except immediately following a previous copulation at least during the period of our observations (June and July). Although a female would copulate with another male within 5 minutes after a pairing, males for the first few minutes showed no interest in a second receptive female even though she would approach and push under the male's cephalothorax. Most males would copulate again after approximately 20 minutes, but two males still showed no interest in receptive females 24 hours after a copulation. In one instance (out of 12 trials) a male showed no interest in a receptive female even though he had no previous contact with a female of any sort for a week. This was not a function of the female, for she copulated immediately with a second male. The reasons for the first male's behavior are unclear; it did not seem to be connected with a pre-molt period. This male did copulate several days later.

In an effort to determine if males would attempt to copulate with non-receptive females which were prevented from attacking, six such females were anesthetized in a solution of 2% ether in sea water and males placed with them. In two cases the males completed the whole courtship sequence except copulation itself; this failure to copulate was apparently because the males were unable to turn the females over and were thus unable to bring their gonopods up to the female's genital opening. In a third instance the male undertook the initial stages of courtship up to palpation of the female's body with the antennules, but then broke off the encounter and paid no further attention to the female. In the remaining cases, there was no obvious courtship behavior although some contacts involved antennular palpation which may have been incipient sexual activity.

Egg-laying and maternal behavior

Following copulation the male and female share a chamber until the eggs are laid. Further copulations may take place during this period. The interval between first copulation and egg-laying was about 10 days at a water temperature of 24–26°. This is the only time when 2 animals share a cavity as indicated by data from both field and laboratory. For example, in the field we collected about 200 animals in the two summers of 1966 and 1968 and found only 4 cavities occupied by 2 animals. In all 4 cases they were male and female, and the ovary-hepatopancreas complex of the female was dark and swollen; also in all 4 cases the female laid eggs shortly after capture. In the laboratory, the male and female jointly occupy a chamber when provided with one and do not engage in aggressive interactions with each other. Both, however, defend the chamber against other stomatopods of both sexes in the period before the eggs are laid. Within 24 hours after laying, the female evicts the male and prevents his return by the appropriate aggressive behavior. Under laboratory conditions, the male may be able to re-occupy the chamber since in the aquaria where the animals were housed, there was usually no other cavity available. The female's aggressiveness increases, however, until the male is no longer able to re-occupy unless he is conspicuously larger than she is in which case he may evict her. In the field evicted males evidently do not return to the chamber since a male was never found in a chamber with a female attending eggs or young. The course of post-laying aggression in one female is indicated in Figure 3; this female was captured with a male but was kept alone with her eggs after laying and males were introduced at intervals. The increase in aggressive behavior is obvious.

Once the eggs are laid and the male evicted, the female remains in the chamber with the eggs and after hatching with the larvae. She frequently palpates the egg mass or holds it with her maxillipeds slowly turning it over. This behavior apparently serves to keep the eggs clean since several isolated masses became contaminated with what are evidently ciliates (R. Kinzie, University of Hawaii, personal communication) and some algae. When defending the cavity, the female leaves the eggs in the recesses of the chamber and comes to the entrance. During this defense she may coil and back into the eggs; this has led to the erroneous statement, originated by Brooks (1886), that the females carry the egg mass over the back (*cf.* Barnes, 1968). The larvae when they hatch are photonegative and attach themselves to any surface they encounter and thus are behaviorally adapted

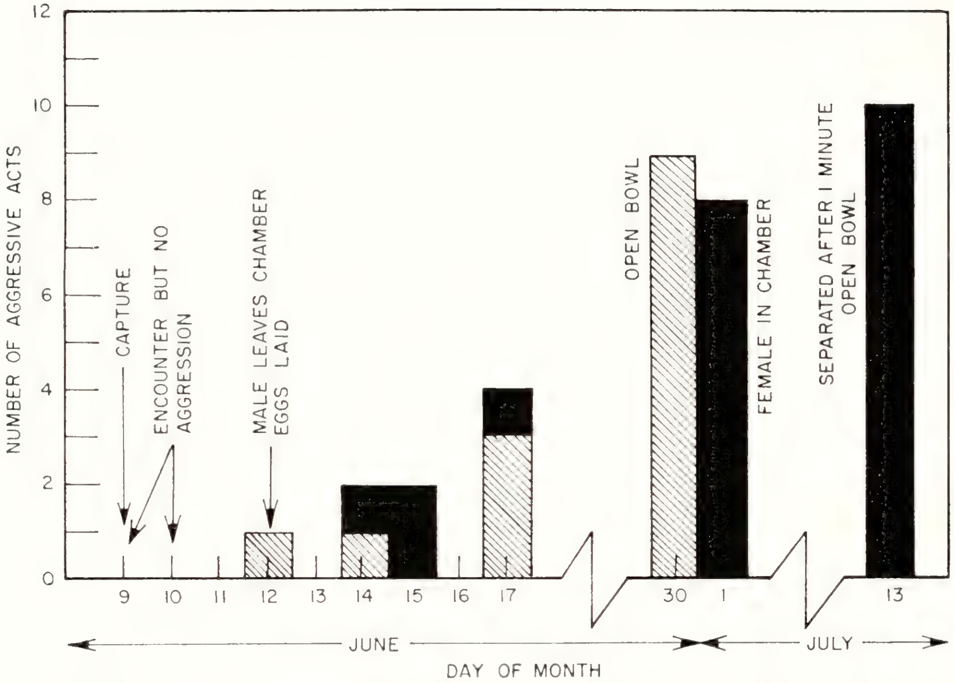

FIGURE 3. Increase in aggressive tendency of a female following egg-laying. Interactions were observed between this female and the male captured in the cavity with her, except for July 1 when a strange male was introduced. Hatched bars indicate number of aggressive acts directed toward a male that did not involve a strike; solid bars indicate strikes. "Open bowl" indicates interactions when the female was removed from her cavity and placed with a male; "female in chamber" indicates interactions when the female was in her "home" cavity, and a male was introduced into the bowl containing the cavity. Brief open bowl encounters were observed on June 9 and 10 when both animals were outside the chamber. On June 12 eggs were laid, and the male left the chamber and was removed to another bowl.

to remaining in the chamber (Dingle, 1969b). The female remains with them until the molt to Stage IV at which point they become strongly photopositive and leave the chamber to enter the plankton. The first 4 larval stages are described by Manning and Provenzano (1963). The period from the time the eggs are laid until the time the larvae enter the plankton is approximately 30–35 days at 24–26° C. On two occasions females with eggs were noted outside the chamber, but returned to it immediately when approached. The egg mass was left in the chamber during an excursion.

Both males and non-receptive females will carry an egg mass if presented with it, but usually drop it after a short period or begin to eat the eggs. They are especially likely to eat the eggs if they become separated from the matrix that maintains the cohesiveness of the egg mass. Males seem particularly inclined to eat eggs, and this is probably a contributing factor to the eviction of the male following egg-laying.

Discussion

When both male and female specimens of *Gonodactylus bredini* are receptive, mating takes place rapidly and the associated behavior is relatively simple and straightforward. The entire sequence from first encounter to release of the female following copulation can take place in between one and two minutes. Reproductive behavior in stomatopods contrasts with the highly complex mating behavior and pair formation observed in many other Crustacea (Schöne, 1961, 1968; Hazlett, 1966, 1968; Johnson, 1969; Atema and Engstrom, 1971) and resembles the situation described, for example, in the spider crab, *Libinia emarginata* (Hinsch, 1968). Also of interest is the fact that there is no aggressive behavior prior to mating even though aggressive behavior in non-sexual situations is very marked in *G. bredini.* Indeed non-receptive females attack males almost immediately.

The absence of aggressive displays during sexual encounters implies that there are precise signals allowing for rapid recognition. In females readiness to mate may be signaled by failure to attack. This is suggested by two results. First, when males were placed with non-receptive females, it was invariably the females which attacked first. Secondly, on 3 of 6 occasions, males displayed sexual behavior toward anesthetized females which had previously been shown to be non-receptive (*i.e.*, they did not possess dark telsons (Fig. 2), and they attacked males). In this *G. bredini* resembles some gammarids and decapods, where any individual which does not resist is seized and treated as a female (Schöne, 1961). The signals from a female which initiates mating are obvious since she approaches and forces her head under the male's carapace; in the same situation an aggressive female approaches far more rapidly, usually with the raptorial meri widely extended laterally, and invariably strikes. A role for the female as the initiator of sexual behavior is somewhat unusual, but not unknown among Crustacea (Schöne, 1961). Limited observations suggest that such a role is even more marked in another gonodactylid, *Pseudosquilla ciliata* (Caldwell and Dingle, unpublished observations).

Whatever else may determine receptivity in *G. bredini,* the molt is evidently not involved. In this, *G. bredini* is like several other Crustacea representing diverse groups, *e.g.*, xanthid crabs (Knudsen, 1960), hermit crabs (Hazlett, 1968), and majid crabs (Hinsch, 1968). It seems evident that mating systems independent of molting are by no means uncommon in crustaceans and may in fact occur as frequently as the molt-associated systems once thought to be the predominant mode (Schöne, 1961). In *G. bredini* a receptive female, while not soft from a molt, is still distinguishable morphologically because of the dark telson.

The absence of molt-associated mating raises the question of pheromones since these substances have long been thought to be associated with molting individuals and have recently been demonstrated in mating (Ryan, 1966; Atema and Engstrom, 1971). We have not demonstrated a pheromone in *G. bredini* courtship nor have we seen any evidence to suggest one. This, of course, does not mean one is not present although we think it unlikely. Mating takes place so rapidly and the non-pheromonal signals seem so clear, *e.g.*, a receptive female does not immediately attack, that a pheromone seems unnecessary for successful mating.

Finally, the relatively simple mating behavior but relatively complex prespawning and maternal behavior of *G. bredini* would seem to be adaptations to cavity living. Burrow or cavity living with associated defensive behavior would afford protection to developing eggs and young. It would not, however, favor elaborate courtship involving visual displays, fencing, circling, etc. (*e.g.*, Hazlett, 1966; Johnson, 1969; Atema and Engstrom, 1971); this is because the cavity is dark, thus limiting vision, and constrained, thus limiting movement. The only visual element in the sexual behavior of *G. bredini* is the approach (usually by the male) with some spreading of the meri; in nature this undoubtedly takes place at the cavity entrance. A female can identify an entering stranger and a male can identify a receptive female in the same way: an appropriate partner approaches slowly or accepts an advance while an inappropriate one attacks. The remaining behavior seems to involve predominantly tactile inputs and is performed with an economy of movements as the male works his way up the back of the female and finally copulates with her. The joint occupation of a cavity by a male and female prior to spawning probably evolved for two reasons: it is advantageous for the male to insure that he alone fertilizes a given egg mass, and for the female because it provides for additional defense during the spawning period. In sum the reproductive behavior of *G. bredini* seems to reflect the co-evolution of four elements: cavity dwelling, defense of the cavity, courtship and copulation, and maternal care. The same is undoubtedly true for *Haptosquilla glyptocercus* and other Gonodactylidae because both Serène's (1954) observations and our own (unpublished) indicate that the behavioral repertoire of these species is very similar to that of *G. bredini.*

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SUMMARY

1. Courtship and copulation are brief in *G. bredini.* Either sex can indicate courtship by approaching; after antennular palpation the male then grasps the female by the carapace and copulation takes place.

2. Receptive females can be distinguished morphologically by darkened internal organs visible through the telson and behaviorally by their acceptance of a male's approach. Unreceptive females attack males immediately.

3. Males share cavities with females during the few days prior to spawning. Following egg-laying females defend cavities against males. The eggs are kept in the cavity and frequently manipulated; the newly hatched larvae remain in the cavity with the female until they reach Stage IV.

4. The form and duration of reproductive and maternal behavior in *G. bredini* are apparently a consequence of cavity living.

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Reference: *Biol. Bull.,* **142**:427–445. (June, 1972)

IN VIVO INTESTINAL ABSORPTION OF SUGAR IN THE TOADFISH (MARINE TELEOST, *OPSANUS TAU*)

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Previous studies on the intestinal absorption and transport of nutrients in fish have been generally restricted to *in vitro* experiments. In such investigations everted or uneverted sacs or segments of intestinal tissue have been used to examine the mechanisms of sugar, amino acid, ion, and water transport (Aull, 1966; Carlisky and Huang, 1962; House and Green, 1965; Huang and Rout, 1967; Huang and Chen, 1971; Mepham and Smith, 1966; Musacchia, Westhoff and Van Haaren, 1966; Neff and Musacchia, 1967; Read, 1967; Rout, Lin and Huang, 1965; Sharratt, Bellamy and Jones, 1964; M. W. Smith, 1966, 1967; R. L. Smith, 1969; Stokes and Fromm, 1964; and Wilson, 1957).

The role of the intestine in ionic and osmotic regulation of fish has usually been examined under well defined *in vivo* conditions (Dall and Milward, 1969; Evans, 1967; Maetz, 1970; Potts, Foster, Rudy and Howell, 1967; Potts and Evans, 1967; Skadahauge, 1969; and Shehadeh and Gordon, 1969). However, *in vivo* investigations of the intestinal absorption of sugars and amino acids in fish have been limited to the early experiments of Cordier and his associates (Cordier and Channel, 1953; and Cordier, Maurice and Worbe, 1957) and Buclon and Peres (1963). Unfortunately these studies were not executed under rigorously defined conditions. For example, net transfer of water from the lumen was not measured. Since the intestinal absorption of water is appreciable when fish are kept in sea water, it can substantially influence the measurements of net solute transfer in the intestine (Maetz, 1970; Potts and Evans, 1967; and Skadahauge, 1969). Other objections to the earlier experiments cited include: (1) Sugar and amino acid solutions directly introduced into the intestine were not saline solutions and their osmolarities were often unknown. (2) The concentrations of substrates were unusually high (Cordier, Maurice and Worbe, 1957) and the time course of the experiments arbitrarily long. (3) The absorptive region of the intestine could not be defined and controlled due to the inadequacy of the technique employed by these investigators.

The present paper describes a rigorously defined technique for the study of intestinal absorption of nutrients in fish under *in vivo* free swimming conditions. This procedure is not only free of the above mentioned deficiencies but will permit rather precise measurements of the absorption rates of various nutrients under physiological conditions closely approximating those in nature. Consideration of natural physiological conditions are particularly important in cases where *in vitro* studies have led to enigmatic results. For example, flounder, trout, and toadfish maintain blood sugar levels which may be higher than 30 mg%, yet attempts to show active transport of sugars by *in vitro* intestinal segments of these fish have failed (Nace, Monle and Schuh, 1963; Rout, Lin and Huang, 1965; Stokes and Fromm, 1964; and Wilson, 1957).

This *in vivo* approach also permits a new evaluation of the "sodium-coupled transport" mechanism proposed by several investigators (see Crane, 1968; Fordtran and Rector, 1971; and Schultz and Curran, 1970). Preliminary reports of this work have already been presented (Farmanfarmaian, 1971 and Farmanfarmaian, Ross and Mazal, 1971). The details will be reported in another communication.

Methods and Materials

Animal collection

Specimens of toadfish (*Opsanus tau*), weighing 150–380 g, were obtained from the Supply Department of the Marine Biological Laboratory (MBL). These animals were collected by local fishermen in the shallow waters of lower Cape Cod in the spring.

The collection procedure is based upon the mating and brooding habits of the toadfish. Empty cans are placed in shallow bays in the spring. During May and June paired toadfish choose these cans as nest sites. The female lays the eggs in the nest and departs while the male remains in or near the nest to guard it until the young leave (Gray and Winn, 1961). The fishermen collect the cans during the guarding period which produces only spent males. If during the same period baited traps are used, the collection will produce mainly spent females since the males do not wander far from the nest. During late summer and fall baited trap collections usually produce a mix of both sexes in various stages of gonad development. The latter have enlarged livers indicating active feeding and storage during the summer.

In this project toadfish were used in all seasons. Repetition of baseline measurements of glucose absorption rate showed no significant ($P < 0.05$) difference between sexes, seasons, or conditions of gonad and liver. Nonetheless, baseline rates were repeated for each new collection of fish.

Animal maintenance

The animals were initially maintained in the large running sea water tanks of the MBL Supply Department at approximately 15° C and were fed live killifish (*Fundulus*) *ad libitum.*

From these stock tanks, healthy animals were transfered to smaller laboratory tanks and acclimated at 20° C ± 1 for about one week. The feeding regimen was as above but each fish was under close observation during this period. All fish that exhibited abnormal feeding or swimming behavior or showed any external wounds and infections were eliminated.

Finally, the fish were starved for approximately 48 hours before an experiment.

The animals used during fall and winter at Rutgers University were shipped from the MBL Supply Department holding tanks by bus or car. They usually arrived in good condition after 6–7 hours of transit in well oxygenated sea water of 5–15° C. In the laboratory 20 animals were placed in each 35 gallon holding tank. The sea water was made from Rila Marine Mix (Rila Products, Teaneck, New Jersey) and its final specific gravity adjusted to 1.025. Each holding tank was fitted with two large Dynaflow pump-filter systems (Metaframe Company, Maywood, New Jersey). Each of these units recirculated the sea water through the glasswool-activated-charcoal filter at a rate of about 4 liters per minute. Filters were changed several times a week as needed. In addition the tanks were aerated through cotton filters by means of separate air pumps. These tanks were in a cold room at 15° C ± 1 and on a 12 hour light-dark cycle. The toadfish were maintained under the same diet regimen and observation indicated above. Live trout fingerlings were used as food during the winter when specimens of *Fundulus* were not available. The animals were then acclimated for about one week at 20° C ± 1. Other conditions were similar to those described above. These toadfish were also starved for about 48 hours before surgery.

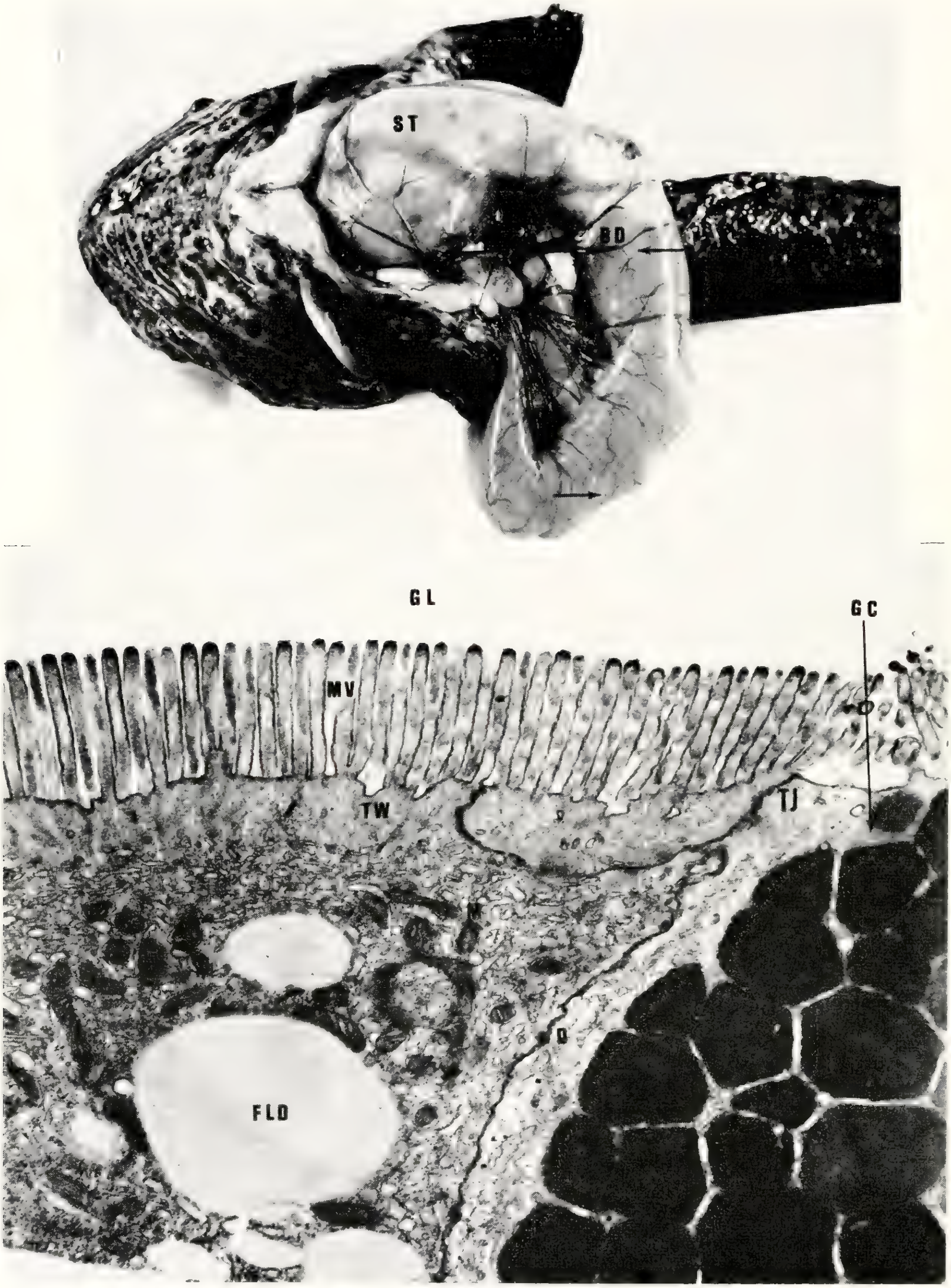
In all cases experiments were performed in healthy animals which had empty stomachs and upper intestines but showed gray or white fecal matter in the lower part of the gut. Unless otherwise indicated, all the experiments were carried out at 20° ± 1.

Surgical operations

The experimental fish was transfered from the starvation tank into a plastic bucket containing a "knock-out" anesthetic solution. This solution was prepared by dissolving 0.5 g of MS-222 (ethyl m-aminobenzoate methanesulfonate, Sigma) in a liter of sea water. When the opercular movements became reduced and the animal could not right itself, it was removed, drained on paper towel, and weighed. The toadfish was then placed upon a sponge covered V-shaped fish rack in a supine position. The rack was in a plastic pan containing a maintenance anesthetic solution (0.1 g MS-222/liter sea water). The solution was siphoned into a Dynaflow pump chamber where it was saturated with oxygen and then pumped through a Y-tube placed in the mouth of the fish over the gill arches. The solution returned to the pan by gravity flow. The pump maintained a flow of about 1 liter/minute over the gills. Toadfish could be kept under anesthesia in this manner for several hours without harm. The heart beat could be visually observed or palpated ventrally just anterior to the pectoral girdle.

When the fish showed no movements other than occasional opercular contractions, an incision was made 2–3 cm anterior to the anus and extended to within one cm of the pectoral girdle. The body walls were retracted and the viscera exposed. These operations resulted in little or no bleeding if the iliac vessels near the anal region remained intact.

The intestine (midgut) was brought into full view and the common bile duct was located. The position of the bile duct in a well fed animal is illustrated in Figure 1. A 2 mm incision was made in the wall of the intestine at a point opposite to and just below the entrance of the bile duct (Fig. 1, anterior arrow). A tightly capped trocar was placed into the incision and its tip maneuvered in the posterior direction into the lumen. A ligature secured the trocar in place and simultaneously closed the intestine just below the entrance of the bile duct. At the lower extremity of the intestine a half centimeter incision was made and a large glass cannula, attached to a drainage tubing, was inserted into this slit. The lumen was washed by delivering three 5 ml portions of the desired saline


ST
BD
GL
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MV
TW
TJ
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FLD

FIGURES 1–2.

in a syringe through the trocar. The wash fluid was drained out by gently lifting the gut. Finally, a second ligature was used to tie the posterior part of the experimental *in situ* gut loop at 6–7 cm below the trocar (Fig. 1, posterior arrow). The blood vessels were carefully avoided in these operations so that there was no bleeding and the circulation to the loop was intact. Furthermore such isolated loops were observed to undergo tonic and rhythmic contractions.

The desired test solution was directly intubated into the lumen of the loop through the trocar and the volume was adjusted so that the gut would be fully extended but exhibit little or no positive hydrostatic pressure. This volume was usually 4–5 ml. The loop was then gently shifted back and forth to mix and equilibrate the luminal contents rapidly. The trocar was opened and an initial sample of about 0.4–0.8 ml was drawn into a 1 ml syringe (graduated in 0.01 ml units) and the cap replaced. This sampling marked the starting time of the absorption period. The retractors were then removed and the abdomen rapidly closed with 9 mm Michel clips (Clay-Adams). The total surgical time was 7 ± 1 minutes. The fish was transferred to an oxygenated sea water tank and moved about to provide flow of sea water through the mouth over the gill arches. This resuscitation was maintained for a minute at which time the animal usually showed signs of revival (opercular and tail movements). In a few cases resuscitation extended over 2–4 minutes. The revived fish swam quietly in this tank and in all respects appeared normal (such animals could be kept alive for 8 days). Twenty-five minutes after the initial sample was taken, the fish was again transferred to the "knock-out" anesthetic solution. After complete anesthesia, the animal was returned to the fish rack and the abdomen rapidly opened. A 1.0 ml terminal sample was removed through the trocar and the time noted. The "incubation" or absorption period was 30 ± 1 minutes unless otherwise indicated. The experimental loop was excised by transversely cutting beyond the ligatures and stripping off the mesenteries. The external fluids were wiped off with tissue paper and the loop was inspected for leaks. Next the lower ligature was cut off over a wide mouth graduated centrifuge tube. The remaining luminal fluid was thus collected and its volume was recorded. The upper ligature and the tissue beyond it were trimmed off and the remaining true absorptive tissue was drained upon Whatman No. 1 filter paper. The wet weight of this tissue was determined on a Mettler H-10T balance. The tissue weights ranged between 1.5–3.5 g. Then the tissue was transfered either to iced 10% TCA for further analyses or discarded. The animal was sacrificed by excision of the heart under anesthesia.

FIGURE 1. Illustration of viscera in a freshly dissected fed toadfish. This animal weighed 198 g and contained 39 g of *Fundulus* which had been made available during the preceding 24 hours. The photograph shows the food in the stomach (ST) and the upper extremity of the intestine. Note the bile duct (BD) which serves as an anatomical marker for the insertion of the trocar. The arrows mark the limits of experimental loop. This region of the intestine is particularly marked by extensive circulation. Magnification is ½ of natural size.

FIGURE 2. Electron micrograph showing the microvilli (MV) of an absorptive epithelial cell extending into the gut lumen (GL). Tight junctions (TJ) can be observed in the region of the terminal web (TW) where neighboring epithelial or goblet cell (GC) adhere together. Below the terminal web area mitochondria (M) and free lipid droplets (FLD) may be observed. The electron micrograph was kindly provided by Dr. M. L. Cayer. Magnification is 14,000 ×.

Solutions

The physiological salt solution used in these investigations was similar to the marine teleost Ringer developed by Forster and Taggart (1950) and therefore will be referred to as "FTR." The final millimolar concentration of the solutes in this saline were: NaCl, 134; KCl, 2.5; $CaCl_2 \cdot 2H_2O$, 1.5; $MgCl_2 \cdot 6H_2O$, 1.0; NaH_2PO_4, 0.5; and $NaHCO_3$, 15.0. The osmolarity of the FTR is approximately 280 milliosmoles per liter. This saline was gassed with 95% O_2–5% CO_2 before use in order to oxygenate and stabilize the pH at 7.2–7.3.

Test solutions containing D-glucose, inulin or other solutes were prepared in FTR. Tritiated or "cold" inulin (5000 MW, New England Nuclear Corp. and Fisher Scientific Company, respectively) was used to measure fluxes of water and the tissue extracellular space. The ratio of the terminal concentration to the initial concentration of inulin was used to correct changes in glucose concentration which resulted from changes in lumen water volume.

Chemical analyses

Glucose was measured colorimetrically by a highly purified glucose oxidase (Glucostat Special, Worthington Biochemical Corp.). The reagent was prepared in 0.2 M pH 7.0 Tris buffer (Sigma Chemical Co.). The procedures were similar to those described in earlier papers (Farmanfarmaian, 1969a and 1969b).

Inulin was colorimetrically determined by a modification of the method of Heyrovsky (1956). It was found that the purple violet color of this 3-indoleacetic acid reaction is stable at 530 nm for an hour if the reaction tubes are incubated for 80 minutes at 37° C and then equilibrated for 20 minutes at room temperature.

The water content of intestinal tissue was determined from the difference in the weight of the drained fresh tissue and its constant dry weight.

Radioisotope procedures

All radiochemicals were purchased from New England Nuclear Corporation. Radioactivity was measured by means of a Packard or a Picker liquid scintillation counter. The procedures for these measurements as well as thin layer chromatographic identification of labeled compounds was described previously (Farmanfarmaian, 1969b).

Histological preparation

Mucosal tissues from the experimental region of the intestine were fixed in 3% glutaraldehyde phosphate buffer at pH 7.0. This was followed by postfixation in 1% osmium tetroxide-phosphate buffer pH 7.0. The tissues were then dehydrated in acetone, embedded in Spurr's medium and examined on a Hitachi-8-1 electron microscope.

Treatment of data

Data are reported as mean ± the standard error at $P \leq 0.05$ (N) unless otherwise indicated. Computations related to the radiotracer method were in accordance with Wang and Willis (1965). Statistical treatment of the data was in accordance with procedures found in Mather (1951). Unless otherwise specified absorption or transport rates are expressed in units of μmoles/g fresh intestinal tissue/hour.

RESULTS

Anatomical and histological observations

In the toadfish the gastrointestinal tract consists of a short esophagus extending from the oropharyngeal sphincter to a thick walled stomach which is slightly J-shaped (Fig. 1). The small intestine or midgut extends from the pyloric constriction to the short hindgut near the anus. The intestine is essentially a straight tube, lacking any of the diverticulae present in other teleosts. It is 1–3 cm in diameter and 12–18 cm in length. It receives its blood supply mainly from the mesenteric artery and is drained by the hepatic portal vein.

TABLE I

Effect of MS-222 anesthesia on the intestinal absorption of D-Glucose in the toadfish, Opsanus tau

	Initial glucose conc. mM	Absorption rate μmoles/g tissue/hour	Terminal intestinal tissue conc. μmoles/ml tissue H_2O
Anesthetized during absorption	10	6.83 ± 0.81 (11)*	5.72 ± 1.0 (11)*
Swimming during absorption	10	6.86 ± 1.17 (6)	3.42 ± 0.98 (6)

* values are means ± SE (N) at $P \leq 0.05$.

Histological sections examined by light microscopy were generally similar to those of mammals except that the folds of mucosa extending into the lumen were thicker and more extensive (Bloom and Fawcett, 1968). The microanatomy of the mucosal epithelium was examined by electron microscopy. The absorptive epithelial cell is about 20 μ in height and has an average diameter of 5 μ. Its luminal border is covered with microvilli which are about 1 μ long and 0.1 μ in diameter (Fig. 2). At a magnification of 50,000 × delicate filaments covering the microvilli are discernible. These have been previously observed on cat microvilli and referred to as "fuzz" by Ito (1965). In the area of the terminal web at the sides of the cells the outer membranes form a junctional complex including tight junction, intermediate junction, and desmosomes. Below the terminal web other cell organelles, such as lysosomes, mitochondria, reticulum, and nucleus, much like those described for mammals, can be seen.

In summary, the toadfish intestinal mucosa is covered by an absorptive epithelial cell layer which exhibits anatomical features similar to those observed in the well known absorptive epithelium of the mammalian intestine.

Validation of methodology

A number of experiments were designed to test and establish the validity of the analytical and experimental procedures adopted here.

In a series of toadfish the intestinal loop was filled with FTR alone in order to test the effect of the saline on endogenous constituents such as water, glucose, and substances which give a positive Heyrovsky reaction (see methods and materials section). After 30 minutes under free swimming conditions no glucose or Heyrovsky reactive material could be detected in the lumen fluid. Analysis of the tissue showed only a slight reaction to the Heyrovsky test but the mean glucose concentration in micromoles per milliliter of tissue water was appreciable, 1.22 ± 0.13 (5). Tissue water expressed as per cent of standard wet weight was 84.6 ± 0.93 (13).

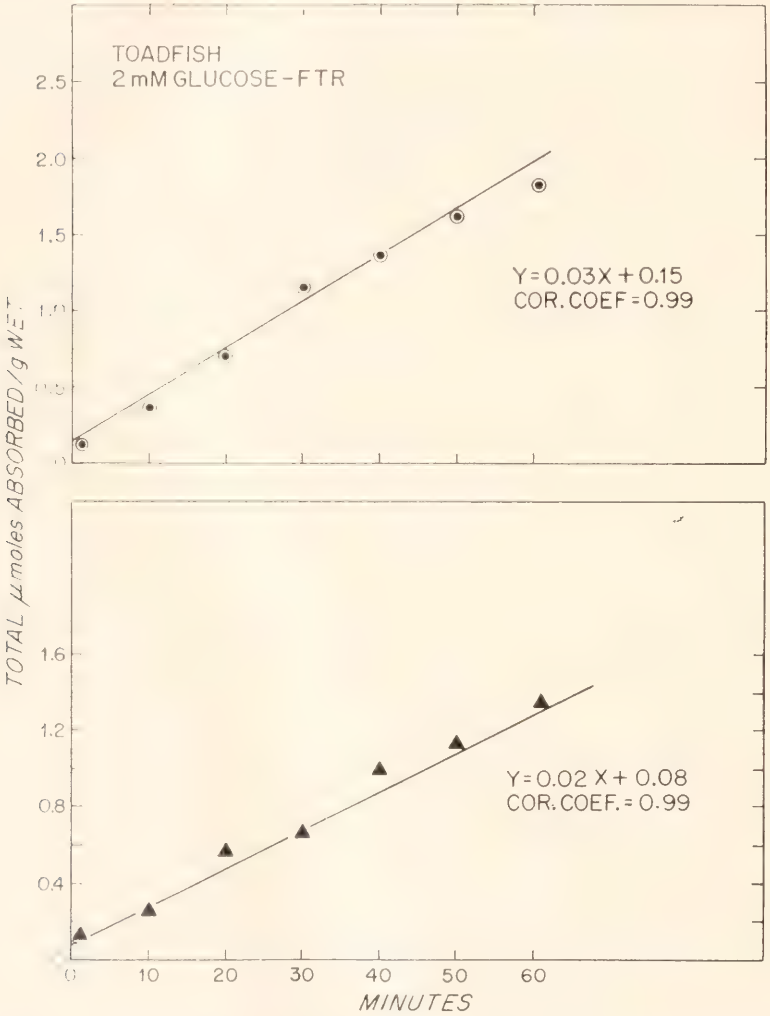

Figure 3. Time dependency of glucose absorption by toadfish intestine *in vivo*. Initial glucose concentration was 2 mM.

The effect of MS-222 anesthesia on the absorption of glucose was investigated in two groups of animals. The first group remained under maintenance anesthesia on the rack while the second group was revived after the operation and allowed to swim during the 30 minute absorption period. In both groups a 10 mM glucose-FTR-^{3}H inulin solution was used. The results reported in Table I show that there is no significant difference in the glucose absorption rate of the two groups. The terminal tissue concentration of the anesthetized group was higher. This is probably due to the supine position under anesthesia which places external pressure upon the veins draining the loop. The resulting reduction in circulation causes a reduced transfer of glucose from the interstitial fluid to the blood. Thus glucose tends to accumulate in the tissue.

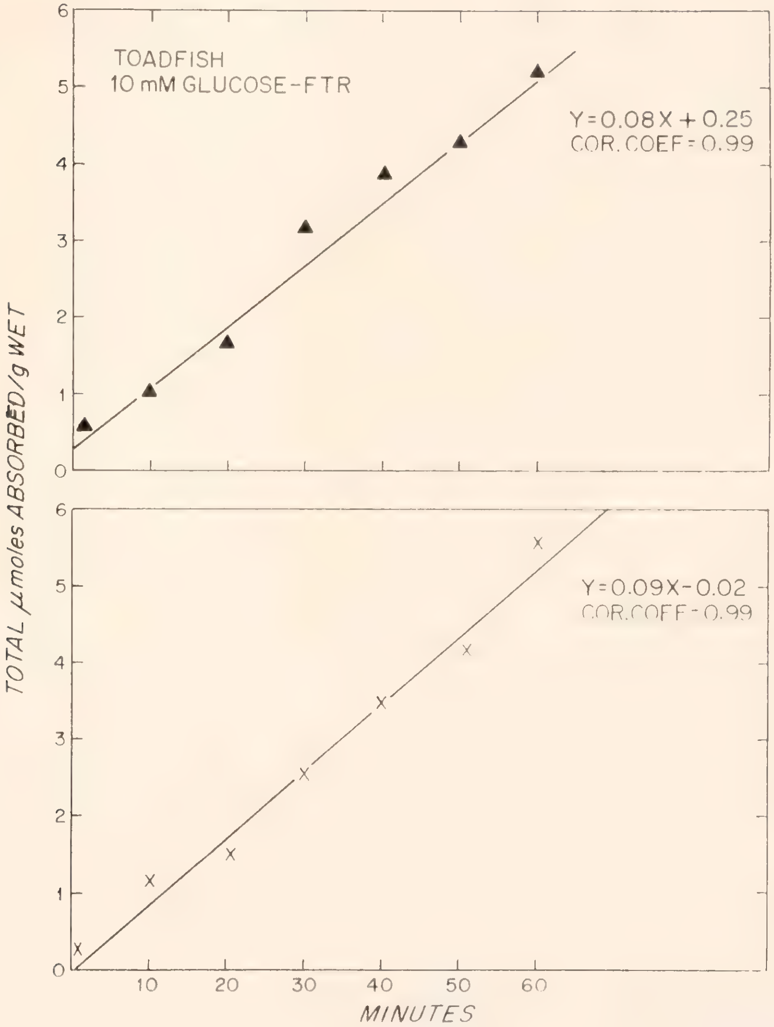

FIGURE 4. Time dependency of glucose absorption at 10 mM initial concentration.

Valid transport rate constants are most conveniently determined in experimental systems where there is a linear relation between the total absorption of substrate and time. Once this linearity is established, a practical time interval (*e.g.*, 30 minutes) may be chosen for kinetic studies. The time dependence of glucose absorption was therefore investigated at 2 and 10 mM concentrations. Samples were removed through the trocar at various time intervals while the fish was under maintenance anesthesia. In these experiments the initial volume of the intubated test solution was usually 5 ml and the sample size was reduced to approximately 0.2 ml in order to sustain a large mass of substrate in the lumen during the experiment. The experiment was repeated for each concentration in 4 or more fish. Typical results are depicted in Figures 3 and 4. The lines are fitted from the calculated regressions but the points are those obtained experimentally. The linear correlation coefficient was higher than 0.96 in all cases and the intercepts were close to zero. For all practical purposes the system behaves like a zero order reaction within the chosen 30 minute limit for incubations. Departure from origin occurred when the test solution was not properly mixed within the lumen at the outset of the experiment.

TABLE II

Absorption of D-Glucose in different regions of the intestine of the toadfish, Opsanus tau*

Fish wet weight (g)	Rate of absorption in μmoles/g tissue/hour		$\frac{\text{Lower} \times 100}{\text{Upper}}$
	Upper third	Lower third	
253	7.08	0.09	1.27
310	3.17	0.63	19.89
382	4.74	0.13	2.75
275	5.48	0.40	7.26
275	6.35	0.89	14.14

* Initial solution was 10 mM glucose-FTR. Both regions were tested in the same fish simultaneously under swimming conditions.

Since different regions of the fish intestine may have different absorptive capacities, it was necessary to establish whether the anterior third of the small intestine chosen for our experiments is in fact the physiologically significant region of normal sugar absorption. The intestine of the toadfish is relatively short, therefore two comparable experimental loops were prepared within the same animal. The upper loop included the first 5 cm of the anterior extremity, and the lower loop consisted of the last 5 cm of the posterior extremity of the intestine. The absorption of glucose was simultaneously determined for both loops in free swimming fish. The results are presented in Table II. In every case the posterior third of the gut had a much lower capacity for glucose absorption in comparison with the anterior third.

Characterization of the glucose transport system

Solute transfer across cell membranes can proceed by one or more of three general processes. These are simple diffusion, facilitated diffusion, and

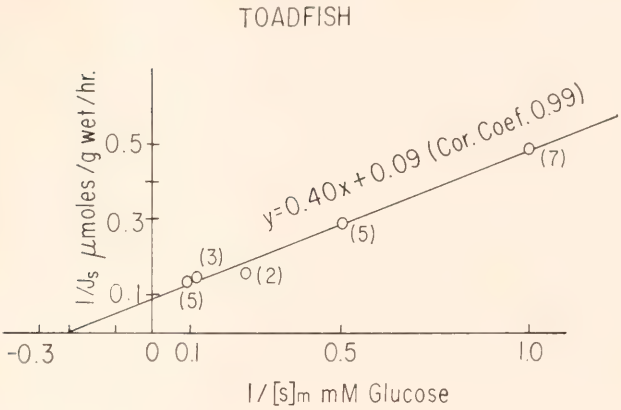

FIGURE 5. Double reciprocal plot showing the relation between net glucose absorption rate and glucose concentration. The line is fitted from the calculated regression but the points represent the actual mean for (N) fish at each concentration.

active transport. The last two are thought to be "carrier mediated." The definition, criteria for recognition, and experimental observation of these processes in various tissues have been reviewed by Stein (1967). The strongest criteria for "carrier mediated" transport under *in vivo* conditions appear to be (1) saturation kinetics and (2) sensitivity to specific inhibitors of the transport system. The phenomenon may be described as active transport if, in addition to the above criteria, it can be shown that the substrate is transported against a chemical (or electrochemical) gradient. We have used all three criteria to characterize the process of glucose absorption in the intestine of the swimming toadfish.

First, the absorption rate of glucose was determined as a function of substrate concentrations between 1 mM and 10 mM. As expected, the transfer of this sugar proved to be a saturation phenomenon conforming to the familiar Michaelis-Menten equation, $v = (V\text{max})[S]/(Km + [S])$. There are three linear transformations of this equation (Dowd and Riggs, 1965). The data are plotted in Figure 5 in accordance with the following transformation using notations which are more suitable to transport studies: $1/J_s = (K_t/J^{max})(1/[S]_m) + (1/J_s^{max})$ where J_s denotes net flux of substrate S and J_s^{max} is the net maximum flux; $[S]_m$ is the mucosal concentration of S and K_t is the substrate concentration at which

TABLE III

Effect of 5×10^{-4} M *phloridzin on the intestinal absorption of D-Glucose in the toadfish,* Opsanus tau, in vivo

Initial glucose Conc. mM	Absorption rate	
	Control	Phloridzin
1.0	2.07 ± 0.2 (4)*	0.13 ± 0.1 (5)
10.0	6.86 ± 1.1 (6)	−0.16 ± 1.7 (4)

* Mean rates are given in μmoles glucose/g tissue/hour ± SE(N) at $P \leq 0.05$.

half net maximal flux is recorded. The other transformations were tested, but yielded no improvement in analysis or representation of the data. The transport constants K_t and J_s^{max} (and their 95% confidence limits) calculated from the regression of the fitted line were 3.7 (3.3–4.1)mM and 9.7(8.4–11.7) μmoles/g tissue/hour.

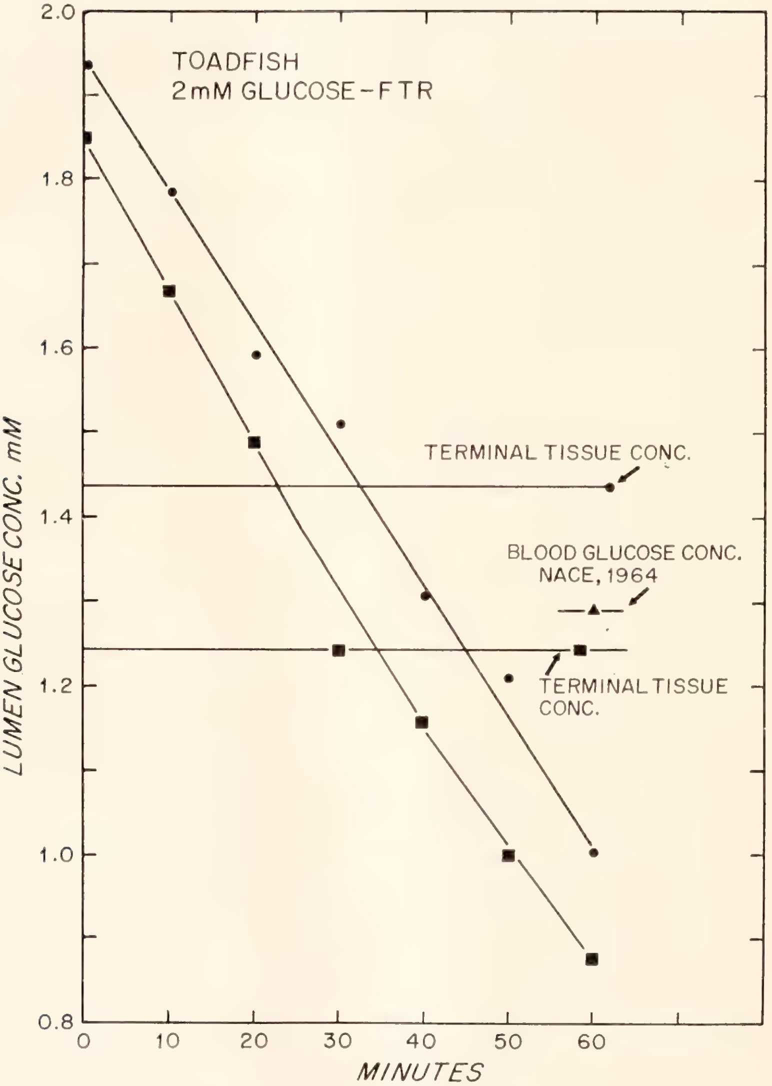

FIGURE 6. Reduction of glucose concentration in the lumen to a level below the tissue and blood glucose concentration due to active transport. The upper horizontal line ● labelled terminal tissue concentration refers to upper absorption curve. The lower horizontal line ■ indicates the terminal tissue concentration for the lower absorption curve. Blood glucose concentration ▲ indicates the mean blood glucose level reported by Nace (1964) for toadfish during the summer season.

Secondly, phloridzin, a well-known inhibitor of the sugar transport system (Malathi and Crane, 1969) was used to test for specific inhibition of this transport system. The results of experiments conducted at 1 mM and 10 mM glucose in presence of 5×10^{-4} M phloridzin are shown in Table III. At both concentration phloridzin abolished glucose absorption. For the higher concentration a slight rise in the luminal glucose was noted (as indicated by the negative absorption rate) in the phloridzin treated animals. Although the magnitude of this rise was not statistically significant ($P \leq 0.05$) in our experiments, the same observation has been reported by others and may prove to be real (Musacchia, Neff and Westhoff, 1964 and Smyth, 1971).

Finally, two types of experiments were done to determine whether intestinal transport of glucose in toadfish can proceed against a glucose concentration gradient under *in vivo* conditions. In contrast with *in vitro* preparations where substrate has a possibility of accumulating in the tissue or on the serosal side, under *in vivo* conditions absorbed substrate is rapidly carried away by blood. Therefore an uphill concentration gradient between the lumen and tissue cannot be observed until the luminal concentration is reduced below the tissue and blood concentration. Hence, *in vivo* experiments which are designed to show active transport should start with a low luminal concentration of the substrate (*e.g.,* twice the homeostatic blood concentration). These considerations are particularly applicable to an animal such as the toadfish in which the maximum velocity (J_g^{max}) for glucose absorption at 20° C is relatively low (9.7 µmoles/g tissue/hour). Accordingly, in the first series of experiments initial luminal glucose was set at 2 mM and absorption was followed at 10 minute intervals for 60 minutes under anesthesia. Tissue glucose concentration was determined immediately after the terminal sample. Typical results are plotted in Figure 6 for two fish. These results clearly show that glucose continues to be absorbed from the lumen solution after its concentration has fallen substantially below that of tissue and blood.

TABLE IV

Absorption and accumulation of D-Glucose in the intestine of the toadfish, Opsanus tau, in vivo

Initial glucose conc. mM	Absorption rate µmoles/g tissue/hour	Terminal intestinal tissue concentration µmoles/ml tissue H_2O	Terminal medium conc. mM	Terminal concentration tissue/medium
1.0	2.07 ± 0.21 (4)*	1.33 ± 0.36 (4)*	0.26 ± 0.16 (4)*	5.3
2.0	3.47 ± 0.62 (5)	1.59 ± 0.58 (5)	1.07 ± 0.22 (5)	1.48
10.0	6.86 ± 1.18 (6)	3.42 ± 0.98 (6)	8.03 ± 0.66 (6)	0.43

* Numbers are mean rates or concentrations ± SE (N) at $P \leq 0.05$.

In the second series, the absorption rate, and the terminal lumen and tissue concentrations were measured for 1 mM, 2 mM, and 10 mM glucose solutions after a 30 minute interval in swimming fish. These measurements as well as the terminal tissue/medium concentration ratios are presented in Table IV. For the 1 mM and 2 mM initial concentrations the tissue/medium ratios are significantly greater than one. These results confirm the uphill transport of glucose against its concentration gradient and therefore the process can be called active transport. Although absorption is high at 10 mM, the incubation time is not sufficient to reduce the concentration in the luminal medium below that of the tissue. The fact that phloridzin completely abolished glucose transport at 10 mM concentration (Table III) is a further indication that nearly all the glucose absorption at this concentration is also by an active transport process.

DISCUSSION

A new method has been described here and applied to demonstrate the active transport of glucose in the intestine of free swimming toadfish. This method may be used to study the intestinal absorption of any nutrient or other substances in fish under well defined conditions approximating the normal physiological state of the animal in nature. The weaknesses inherent in previous *in vivo* investigations of sugar and amino acid transport in fish intestine have been rectified. In previous studies (Cordier and Chanel, 1951; Cordier and Maurice, 1956), the region of the intestine exposed to the nutrient solution could not be specified. These authors injected various solution via the anal pore and measured the absorption of the substrate after a specified time interval. When a solution is injected in this manner, it may pool in the posterior part of the intestine or spread throughout the gastrointestinal tract depending upon the fluid volume and the capacity of the gut. In the present study it has been shown that the rate of absorption of glucose in the lower third of the gut is about 10% that of the upper third of the intestine. Carlisky and Huang (1962) reported that glucose transport in segments of the "duodeno-jejunal" region of the dogfish was higher than the "ileum." Regional specialization of the intestine is also well established for the mammals (Booth, 1968). Therefore, *in vivo* absorption measurements cannot be considered valid unless the specific region of the intestine is clearly defined.

Another inadequacy of previous *in vivo* techniques was that the state of the intestinal contents, including food, fluid, parasites, and bacteria could not be ascertained prior to the experiments. Furthermore, samples could not be taken except at the end of the experiment. Since indicators such as inulin were not used to measure water volume, dilution by extant intestinal fluids erroneously appeared as reduction in substrate concentration. In the present technique the trocar allows for washing the loop of all its previous contents and for sampling at any desired interval. Inclusion of a water indicator was found to be essential since in most of the experiments there was an appreciable absorption of water from FTR solutions, amounting to 2–18% of the initial lumen volume.

The *in vivo* method permits the tonic and rhythmic contractions of the intestine to proceed normally. Therefore the problem of "unstirred layers," which may be a source of error under *in vitro* conditions is minimized. In our attempts to use isolated everted sacs of toadfish in glucose-FTR solutions the intestinal segments underwent severe sustained contractions. No transmural glucose transport could be observed in such preparations. These observations were similar to those reported for toadfish by Wilson (1957), for flounder by Rout, Lin and Huang (1965), and for trout by Stokes and Fromm (1964).

It is difficult to explain such a discrepancy in sugar transport between the *in vitro* and *in vivo* studies. However, any interpretation that rejects the occurrence of intestinal absorption of glucose in these and other fish is physiologically untenable. Most fish have relatively high blood sugar and liver glycogen levels. Nace, Monle and Schuh (1963) reported that the blood glucose of toadfish was about 100 mg% between January and April and 20–30 mg% during the summer. Rout, Lin and Huang (1965) found the blood glucose concentration of the flounder to be between 26–50 mg%. Stimpson (1965) reported a liver glycogen value of 43 mg/g tissue in goldfish, nearly as high as that in rat (47 mg/g). It is unreasonable to suppose that such high levels of blood sugar and liver glycogen in fish, comparable to those of mammals in some cases, are entirely achieved through gluconeogenesis. In studies of the general intermediary metabolism of fish (Hochachka, 1969) there has been no evidence of unusually high gluconeogenetic activity. Absorption routes other than the intestine are unlikely; body and gill surfaces are not highly permeable to sugars and amino acids and the available quantities in the sea are not appreciable, 10^{-7} M (Siegel and Degen, 1966). An effective intestinal sugar transport system is indicated by the finding of efficient utilization of ingested carbohydrates in trout (Phillips, 1969). Therefore it is more reasonable to regard the intestine as the normal rout of sugar absorption and seek other explanations for the failure of its detection in some *in vitro* studies.

Wilson (1957) and Rout, Lin and Huang (1965) reported that intestinal segments of the toadfish and the flounder, which were capable of transmural uphill transport of amino acids, could not transport glucose in the same manner. An explanation for these observations may be found in the reduction of O_2 supply and the consequent Pasteur effect under *in vitro* conditions. Although flasks are meticulously gassed with 95% O_2–5% CO_2 in such experiments, the O_2 capacity of salines (about 0.5 volumes % at 20° C and less at higher temperatures) is a limiting factor. This capacity is adequate for tissues of some invertebrates, such as *Thyone* (Echinodermata), which normally maintain low O_2 tensions in their body fluids (Farmanfarmaian, 1966). But for most vertebrates the O_2 capacity of salines is at least one order of magnitude less than the O_2 capacity of their blood, usually between 6–20 volumes % (Prosser and Brown, 1961). Pietra and Cappelli (1970) have shown that the O_2 consumption of rat intestinal segments does not reach saturation even at pO_2 of 650 mm Hg, the highest pO_2 used. The stimulation of glycolysis, due to inadequate oxygen supply and Pasteur effect, increases the cellular utilization of glucose. For rat as much as 75% and for hamster 45% of absorbed glucose may be converted to lactate in isolated segments of the intestine (Wilson, 1956). In these mammals we find the *in vivo* and *in vitro* glucose absorption rate at 37° C to be several times higher than the swimming fish at 20° C (Farmanfarmaian, unpublished data). Therefore in the case of the mammalian intestinal segments the mass of absorbed glucose may be sufficient to permit mucosal intracellular accumulation and uphill transfer to the serosal side in spite of a high rate of glucose utilization under hypoxic conditions. In fish intestine, on the other hand, the glycolysis rate *in vitro* may be so high relative to absorption rate that the active transport to the serosal side is completely masked. Under these conditions, experimental limitations, such as sensitivity of glucose detection methods and tissue survival, become critical. Even in the rat it is difficult to demonstrate uphill transport of glucose *in vitro* unless the incubation period is extended beyond 30 minutes (Wiseman, 1964). Such extension is eventually limited by desquamation and general tissue aging which reduce the absorption process.

The maximum velocity (J_s^{max}) and the concentration at which half maximal velocity is obtained (K_t) may be used as transport constants for a substrate which is transported by a "carrier-mediated" system conforming to Michaelis-Menten kinetics. These constants are of value for resolving components of transport mechanisms and for comparing data obtained under varying conditions or in different species. If the evolutionary unity observed in fundamental processes such as the genetic code, protein synthesis, and metabolic pathways is also true for the "carrier" mechanism of a widespread natural compound such as glucose, then one might expect the glucose K_t to be of the same order of magnitude in different species and under different physiological conditions.

These expectations are generally supported by the K_t values reported in the literature and our present investigations. When statistical variability for different studies is considered, K_t for the *absorption* of glucose by the intestinal mucosa and other tissues is within the same order of magnitude for a variety of vertebrates as well as invertebrates. In the present study K_t for the intestinal absorption of glucose in the swimming toadfish had a mean value of 3.7 mM (summer of 1970) and 4.1 mM (summer of 1971), the difference falling within the 95% confidence limits. For the hamster intestinal segments the reported K_t values are 1.5 and 2.5 mM (Crane, 1968). *In vitro* determinations of K_t for glucose absorption by sheets of rabbit ileum yielded a value of 5 mM when calculated from short circuit currents (Schultz and Zalusky, 1964) and 1.4 mM when measured as ^{14}C-glucose fluxes (Goldner, Schultz and Curran, 1969). In other mammalian tissues such as red blood cells and kidney slices the most frequent values reported for glucose absorption K_t (but not K_i) are between 1–5 mM (Stein, 1967). Kinetic studies of glucose absorption among invertebrates have been limited to the intestine of *Thyone* (Echinodermata), and the body wall of the rat tapeworm. In *Thyone* intestine, where glucose is absorbed by a facilitated diffusion process, the K_t measured with everted intestinal sacs in artificial sea water at 20° C was 1.6 mM (Farmanfarmaian, 1968). The K_t for body wall uptake by the rat tapeworm *in vitro* at 37° C is also 1.6 mM (Read, 1961).

In contrast to the narrow range for K_t, the reported values for maximum velocity (J_s^{max}) are highly variable. These data indicate that the binding site for glucose in the brush border membrane of the intestinal epithelium (and possibly other tissues) may be similar among species. On the other hand, the actual rates of transport which reflect number of functional sites (and possibly other factors) are highly dependent upon experimental conditions as well as the specific tissues and species. The relative constancy of K_t among organisms having different intestinal ionic concentrations in nature (*e.g.*, *Thyone*, toadfish, and rat) may shed light on the proposed role of Na^+ in sugar transport (Crane, 1968; Schultz and Curran, 1970; and Fordtran and Rector, 1971). The similarity in K_t values cited above would seem to indicate that the properties of the glucose binding site, as reflected by K_t are not significantly affected by changes in the extracellular Na^+, at least over the range occurring naturally. Since the possibility remains that the *luminal* concentration of Na^+ required for normal binding and transport of glucose is well below the level naturally available in the lumen, we have undertaken a systematic *in vivo* investigation of this problem in several species. Our preliminary data indicate that a ten fold reduction in the *luminal* concentration of Na^+ in the swimming toadfish has no appreciable effect on K_t or J_s^{max} of glucose transport (Farmanfarmaian, Ross and Mazal, 1971). Details of this work will be published in a subsequent communication.

Messrs Ross and Mazal were Henry Rutgers honor students who made significant contributions to this research work in their senior year. The senior author is a member of the Bureau of Biological Research, Rutgers University.

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SUMMARY

1. A technique for investigation of nutrient absorption *in vivo* in the intestine of free-swimming fish is described.

2. Glucose absorption occurs primarily in the upper third of toadfish intestine and is linear with time.

3. Anesthetized (MS-222) and free-swimming toadfish were similar with respect to intestinal absorption of glucose.

4. The absorption process exhibited saturation kinetics, conforming to the Michaelis-Menten equation. It is inhibited by phloridzin.

5. Active transport of glucose was demonstrated by the fact that absorption continues after the glucose concentration of the intestinal lumen has fallen below that of intestinal tissue and blood.

6. Difference in the *in vivo* and *in vitro* approaches to the study of intestinal transport and the significance of the observed kinetic parameters are discussed.

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ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF A PEPTIDE WHICH STIMULATES THE HINDGUT OF THE COCKROACH, *LEUCOPHAEA MADERAE* (FABR.)

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Although the corpus cardiacum of insects is known to contain a factor that stimulates the muscles of the heart and alimentary tract (Cameron, 1953), Davey (1962) was the first to describe the effect of homogenates of the corpus cardiacum on the spontaneous contractile activity of the cockroach hindgut. He found that when an isolated hindgut was exposed to 0.025 pairs of corpora cardiaca per milliliter, an increase in tonus, amplitude, frequency and coordination of contractions occurred.

Brown (1965) subsequently succeeded in separating six to nine active substances from extracts of the corpus cardiacum of *Periplaneta americana* L. by means of paper chromatography. He found that two components, Factors P_1 and P_2 were responsible for most, if not all, the effect the crude extracts had on hindgut activity. The two compounds appeared to be peptides and were heat-stable, sensitive to chymotrypsin, and dialyzable. Brown assumed that Factor P_1, by its rate of dialysis, was the larger of the two peptides and was as effective on the hindgut as on the heart; P_2 was a specific activator of the hindgut.

In an earlier paper, we described three materials present in hindgut tissue extracts of *Leucophaea maderae* (Fabr.) that stimulated the muscular activity of isolated hindgut preparations from the same cockroach (Holman and Cook, 1970). Two of the compounds were identified as L-glutamic and L-aspartic acids. An application of either of these acids to an isolated hindgut caused a single slow contraction that was indistinguishable from the response produced by electrical stimulation of the nerves innervating the hindgut. An application of the third material (not identified) resulted in a prolonged and complex stimulation of the hindgut which was similar in character to the response reported by both Davey (1962) and Brown (1965) for corpora cardiaca extracts.

We have attempted to isolate and partially characterize this active compound present in the hindgut extracts with the hope of determining its role in the control of muscular activity in this region.

MATERIAL AND METHODS

Extraction procedure

Extracts were made of organs and body areas from adult cockroaches, *L. maderae* and *P. americana*; from grasshoppers, *Schistocerca vaga vaga* (Scudder);

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from house flies, *Musca domestica* L.; and from fifth-instar larvae of tobacco hornworms, *Manduca sexta* (L.). All insects were reared at this laboratory under controlled conditions.

Dissected organs and body parts were homogenized in 80% ethanol (cockroaches and grasshoppers, 5/ml; fly heads, 50/ml; hornworm hindguts, 2/ml). Following centrifugation, the supernatant was evaporated to dryness with a rotary evaporator at 35° C. The residue was then partioned between equal volumes of ethyl acetate and water (25 ml of each/100 organs). The ethyl acetate was discarded and the aqueous phase lyophilized and stored in the freezer under argon.

Extracts for the centrifuge experiments were prepared by homogenizing heads and hindguts of *L. maderae* in 0.25 M sucrose (0.5 ml/organ). Following centrifugation at 1000 *g* (0° C, 15 min) to remove insoluble debris, the supernatant was re-centrifuged at 20,000 *g* (0° C, 30 min). The second supernatant was lyophilized and prepared for bioassay. The sediment was extracted twice with 5 ml of 80% ethanol which was pooled, evaporated, and prepared for bioassay.

Purification

The biologically active material in the crude extracts was purified by anion- and cation-exchange, gel filtration, and thin-layer chromatography.

Twenty grams of DEAE-Sephadex® (A-25) [mention of a proprietary product in this paper does not imply an endorsement by the U. S. Department of Agriculture.] was allowed to swell in 200 ml 50% acetic acid. The acid was decanted and replaced with fresh acid four times. The gel was then rinsed with water until the pH of the gel solution rose to 4.0, then packed with flow into a 2.5 × 45-cm column and rinsed with 1 liter of distilled water. Samples were applied to and eluted from this column with water. Four 50-ml fractions were collected, lyophilized, and stored in the freezer under argon.

Thirty grams of CM-Sephadex (C-25) was prepared and packed in the 2.5 × 45-cm column in the same fashion as the DEAE-Sephadex except that 0.5 N HCl replaced the 50% acetic acid. Samples were applied to this column in water and eluted from the column by the stepwise addition of water, 0.1 N and 1.0 N acetic acid, 0.01 N and 0.5 N HCl (300 ml each). Later, the samples were eluted with water, 0.01 N and 0.02 N HCl (300 ml each), and the 0.02 N HCl was collected as three 100-ml fractions. All fractions were lyophilized and stored in the freezer under argon.

Both G-10 and G-15 Sephadex gels were prepared in the same manner; 50 grams was stirred with 0.01 N HCl then allowed to settle for five minutes, the HCl (and fines) was decanted and replaced with fresh HCl several times. Then the gel was packed without flow into a 2.5 × 45-cm column. After packing, solvent (0.01 N HCl) was passed through the column until the gel bed stabilized. Samples were applied to the column in 0.5 ml of 0.01 N HCl containing dextran blue (to determine the V_0) and eluted with 0.01 N HCl into 4-ml fractions. The flow rate was 4 ml/cm^2/hr (about 20 ml/hr).

Thin-layer chromatography was carried out on 5 × 20-cm glass plates coated with a 250-μ layer of cellulose. A miscible solvent system (isopropanol:water:acetic acid, 25:10:1) was allowed migrate in the ascending fashion. After drying in the hood, fluorescent spots were visualized under a broad spectrum ultraviolet lamp. Amine-containing areas were visualized with ninhydrin spray followed by heating (110° C).

Various regions of unsprayed chromatograms were scraped, and the cellulose was eluted with 0.01 N HCl. The HCl was evaporated, and the residues were dissolved in saline solution for bioassay.

Stability and sensitivity tests

Biologically active samples eluted from thin-layer plates were prepared for bioassay in screw-topped tubes and immersed in a boiling water bath for 10 minutes. After cooling, they were applied directly to the isolated hindgut preparation. Similar samples were titrated to a pH of 9.0 with 0.01 N NaOH, held for 10 minutes, titrated to pH 7.0 with 0.01 N HCl, and bioassayed.

Saline solutions of chymotrypsin and pronase (a mixture of proteolytic enzymes) were prepared at a concentration of 1 mg/ml and brought to pH 7.5 with 0.01 N NaOH. Samples of the active material in 1 ml saline solution (pH 7.5) were treated with $\frac{1}{2}$ ml of one of the enzyme solutions or with $\frac{1}{2}$ ml pH 7.5 saline solution and incubated for three hours at 37° C. After incubation, the samples were immersed in a boiling water bath for 10 minutes, cooled, and then bioassayed. Tubes containing only pronase or chymotrypsin were treated in the same manner and bioassayed.

Bioassay and various physiological preparations

Hindguts from adult male cockroaches were isolated and prepared for myographic recording as previously described (Holman and Cook, 1970). The methods of Cook, Eraker and Anderson (1969) were used for the bioassay of the foregut. On several occasions, the hindgut of fifth-instar larvae of the tobacco hornworm was isolated and suspended by threads in a chamber in a manner similar to that which was used with the cockroach. The saline solution for the tobacco hornworm consisted of 6 g sodium chloride, 0.3 g potassium chloride, 3.6 g magnesium chloride × 6 H_2O, 0.33 g calcium chloride and 54.4 g sucrose dissolved in 1 liter of water and brought to a pH of 6.5. The lyophilized residues were dissolved in 200 to 500 μl of the appropriate saline solution and adjusted to a pH of 6.5 before they were added to the aerated saline solution in the muscle chamber. Samples were left in contact with the gut from 1 to 5 minutes. The chamber was then rinsed several times and refilled with fresh saline.

Both adult *P. americana* and *L. maderae* were used for bioassays of the heart. The type B preparation described by Yeager (1939) was employed. Following several rinses with saline solution, the spontaneous contractile responses of the heart were registered by placing a glass microelectrode against the ventral lateral surface of the heart. The micropipet was used as a resistance sensor to detect the mechanical movement of the heart (Cook, Long and Owens, 1971). Samples were applied directly to the heart in saline solution (1–2 μl), and the heartbeat was monitored for 3–4 minutes to determine the effect of the applied samples.

A small suction electrode was used to record the electrical activity from the proctodaeal nerve when the terminal ganglion was exposed to the extracted peptide.

The electrode was connected to a preamplifier and an oscilloscope was used to monitor spontaneous neural activity.

Histological and cytological procedures

The *in situ* demonstration of neurosecretion in the hindgut of the cockroach was accomplished by employing the Victoria Blue method of staining described by Dogra and Tandan (1964).

Electron micrographs were made of the proctodaeal nerves obtained from adult male Madeira cockroaches that were perfused during dissection with Millonig's (1962) buffered 4% glutaraldehyde solution (pH 7.4). Fixation of the nerve segments was continued at room temperature for three hours, after which time the nerves were rinsed in buffer for one hour and placed in a 1% solution of OsO_4 for three hours. Acetone was used for dehydration, with the 70% step containing enough uranyl acetate to saturate the solution at room temperature. The nerves were left for three hours in this solution and for 20 minutes in the other dehydration steps of 50%, 95%, and twice in 100% acetone. The nerves were embedded in Araldite (Mollenhouer, 1964) and sectioned with a glass knife. Sections were stained with lead citrate (Reynolds, 1963), and micrographs were taken with an RCA EMU-3C at 50 KV.

RESULTS

Purification

In a previous paper (Holman and Cook, 1970), we were able to demonstrate that an excitatory factor present in hindgut extracts was not retained by the DEAE-Sephadex column. Therefore, to eliminate more of the inactive residue, we increased this column to 20 g (compared with the 10-g column used previously) and collected four 50-ml water fractions. The excitatory material was present in the second of the four water fractions. Residue from this fraction was eluted through a cation exchanger (CM-Sephadex) by the stepwise addition of water, two concentrations of acetic acid, and two concentrations of HCl. Although most of the residue eluted with the early fractions, the active material appeared only in the last fraction. However, we soon discovered that 0.02 HCl removed the active material from the column and that the last 100 ml of this eluate (300 ml total) contained all the excitatory activity. Passage of the active material through a CM-Sephadex column after DEAE-Sephadex treatment did not cause loss of activity.

The excitatory compound was eluted as a single peak from G-10 and G-15 gels just after the dextran blue (V_0) and slightly ahead of reduced glutathione (molecular weight = 311). We estimated the molecular weight of the excitatory compound to be in the 400 to 600 range.

Active material from the CM-Sephadex columns was subjected to thin-layer chromatography on cellulose with the isopropanol system. Bioassay of material eluted from several areas of the plates indicated that the active material moved as a single spot and had an R_f of 0.5. When similar plates were not scraped but were sprayed with ninhydrin reagent, almost all the ninhydrin-positive material was found at a lower R_f. The active area was only weakly positive to ninhydrin,

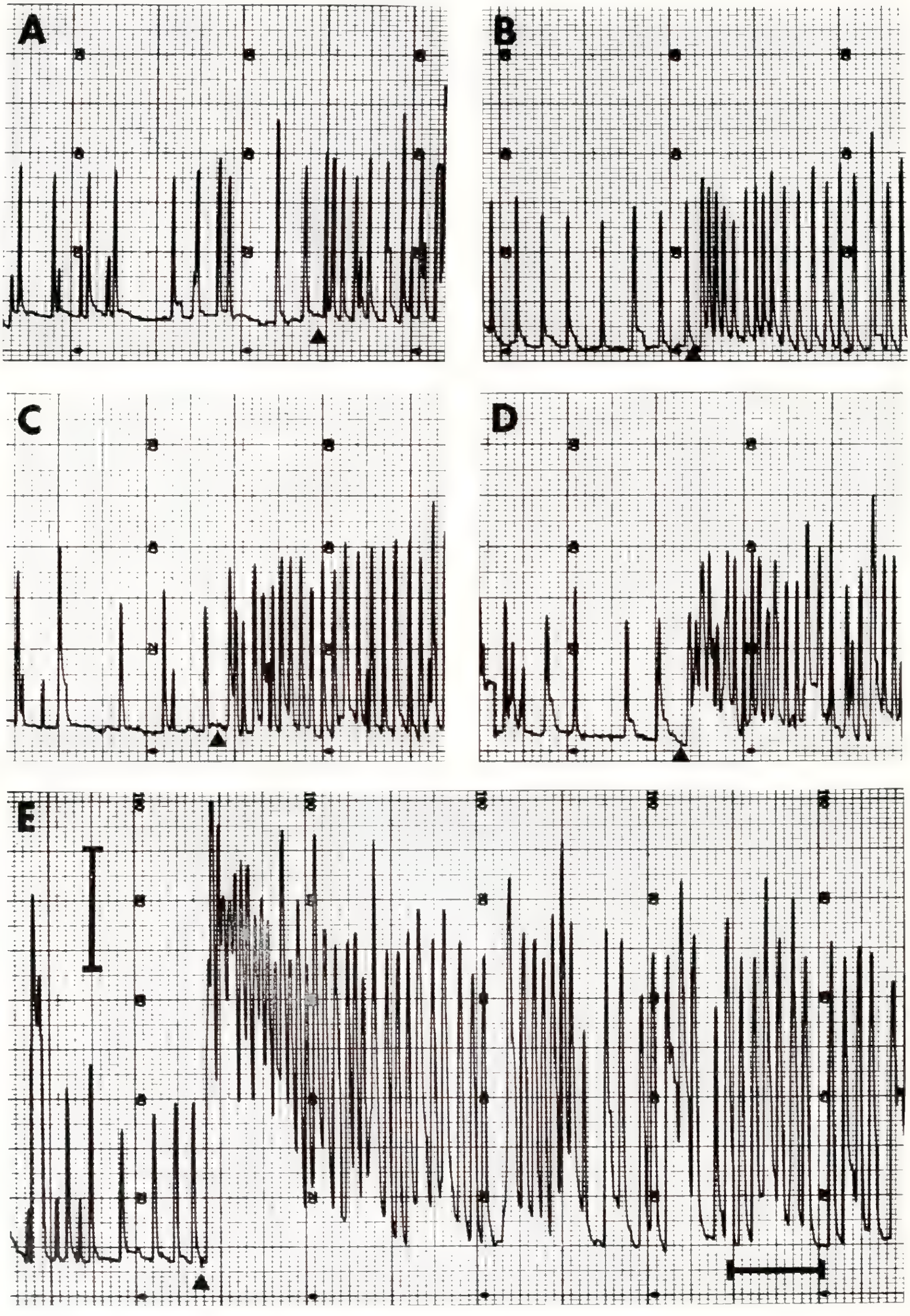

Figure 1. The hindgut myographic response profile to various concentrations of the extracted peptide; hindgut response to (A) 0.05 hindgut equivalents (Heq); (B) 0.12 Heq; (C) 0.25 Heq; (D) 0.50 Heq; and (E) 5 Heq. Triangle marks the addition of the peptide. Bath

and we estimated that only 10 per cent of the ninhydrin-positive material on the plate was associated with this spot.

Sensitivity to heat, pH, and proteolytic enzymes

The biologically active material was stable to heat and alkaline pH under the conditions described in the Methods.

When the active samples were incubated with chymotrypsin for three hours as described, no loss in activity was observed. The solutions incubated with pronase under the same conditions or for 30 minutes lost all biological activity. The enzymatic activity of chymotrypsin and pronase in our system was verified by the digestion of a standard albumin solution.

TABLE I

Distribution and abundance of the stimulatory peptide in the Madeira cockroach

Source of extract	Threshold conc. organ equiv./ml	μg residue/organ after purification	μg residue necessary for threshold response
Head	0.04	0.28	0.011
Foregut	No activity present		
Hindgut	0.004	3.2	0.013
Terminal ganglion	0.25	Not determined	Not determined
Cercal-proctodaeal nerve	0.25	Not determined	Not determined

Species and organ distribution

The stimulatory substance was present in head and hindgut extracts of *L. maderae, P. americana,* and *S. vaga vaga.* It was not present in heads of *M. domestica* nor in the heads and hindguts of fifth-instar larvae of *M. sexta.*

Extracts of foreguts, terminal ganglia, and cercal-proctodaeal nerves of *L. maderae* were processed through the ion exchange columns and bioassayed. Active fractions were obtained from extracts of terminal ganglia and cercal-proctodaeal nerves, but no excitatory activity was present in extracts of the foregut.

Threshold concentrations in terms of the minimum organ equivalents/ml producing the excitatory response are tabulated in Table I. A threshold response is shown in Figure 1a. Residues purified by ion exchange from 100 heads and 100 hindguts were used to determine the values in column 3 of the table. Values shown in column 4 were obtained by multiplying column 2 by column 3. Such minute amounts of residue were present after the purification of ganglia and nerve extracts that accurate weights could not be obtained. Therefore, no values were determined.

Virtually all the biological activity remained in the supernatant when sucrose extracts of heads and hindguts were centrifuged at 1000 *g* for 10 minutes. Recentrifugation of the 1000 *g* supernatant at 20,000 *g* for 30 minutes followed by semi-quantative bioassay of supernatant and sediment (extracted with 80%

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volume was 12 ml, horizontal time mark, 2 min. Vertical calibration refers to 2 mm of actual movement of the hindgut.

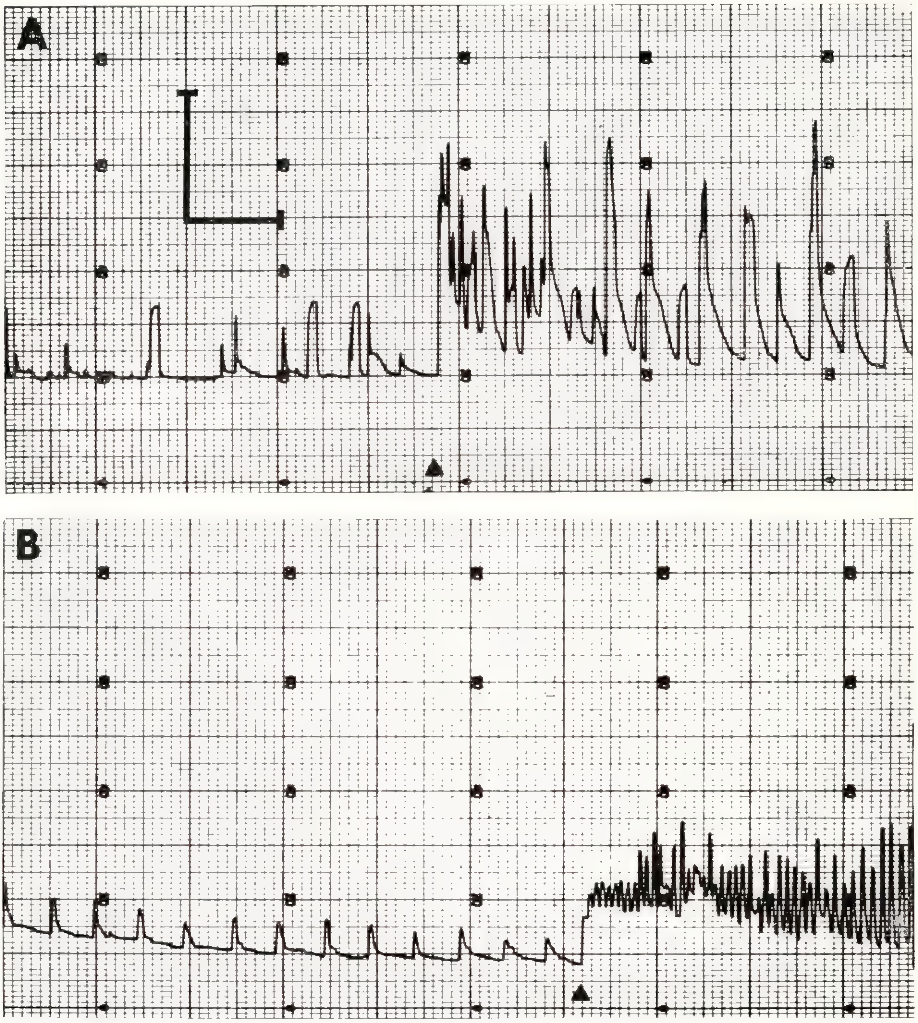

FIGURE 2. Response of the isolated rectum (A) and colon (B) to the excitatory peptide. Triangle marks the addition of 6 head equivalents to muscle chamber; horizontal time mark, 2 min; vertical calibration, 2 mm.

ethanol) showed that 97 per cent of the activity was associated with the sediment, an indication that the stimulatory substance was in the particulate fraction.

Biological properties

The extracted peptide evoked a complex response from the visceral muscles of the hindgut of the Madeira cockroach. Four distinct features were evident on the myographs: (a) an increase in tonus; this was indicated by a rise in the baseline (Fig. 1e); (b) an increase in the frequency of contractions; this was the most evident response obtained from the peptide, even at threshold concentrations (Fig. 1a); (c) an increase in the amplitude of contractions; and (d) an increase in the coordination of contractions; *i.e.*, a greater uniformity in the amplitude, frequency, and contour of contractile events. Only at 0.5 hindgut equivalents or above were all four features consistently present. At tissue concentrations of less than this amount, amplitude and frequency were the only prominent features.

A threshold response was obtained with only 0.004 hindgut equivalents per milliliter or 13 ng of dry residue per milliliter (Fig. 1a). A maximum response was reached with 0.4 hindgut equivalents or 1.3 μg of residue per milliliter (Fig. 1e). The excitation of the hindgut was remarkably persistent. Although not shown in Figure 1e, the response continued for 1 hour and 15 minutes. Even at a concentration of tissue residue of 31 ng/ml, the response persisted for more than six minutes. The presence or absence of the terminal ganglion with its associated motor neurons to the hindgut seemed to have no bearing on the response of the hindgut to the extracted peptide. Indeed, application of the peptide (several hindgut equivalents) to the terminal ganglion produced no change in either the frequency or the amplitude of efferent impulses in the proctodaeal nerve.

Davey (1962) showed that although the addition of homogenates of the corpora cardiaca to the isolated hindgut of the American cockroach produced an increase in tonus, amplitude, frequency, and coordination of contraction, these homogenates would not stimulate the rectum alone. He theorized that the factor in the corpora cardiaca acted by stimulating argentaffin-positive cells in the upper colon to release an indolalkylamine. In turn, this amine was proposed to induce the visceral muscles of the rectum to contract by way of a peripheral nervous system which could function in isolation from the central nervous system.

To test this hypothesis on the Maderia cockroach, we isolated the rectum from the colon and examined the myogenic response of each portion individually. Surprisingly, the extracted peptide caused nearly identical effects on both the rectum and the colon (Fig. 2a, b). The myogenic character of the two separate regions showed all four of the distinctive features mentioned previously, in spite of their individual myogenic rhythms.

Once we suspected that the excitatory substance was a peptide, we began to look for evidence of neurosecretion in the proctodaeal nerve by using Victoria Blue stain. The secertory axons in the proctodaeal nerve were stained in such a way that observations could be made in whole mounts or suitably dissected portions of the bulk-stained preparations. Figure 3b shows a portion of the proctodaeal nerve on the surface of the rectum.

The staining did not reveal continuous pathways coursing through the nerve. Rather, the Victoria Blue-positive material appeared as discrete spots that were arranged in a beaded fashion along the proctodaeal nerve near the surface. This type of staining was evident even in branches of the nerve in the anterior colon. When we observed the nerve in cross-section under the electron microscope, the axons containing the nuerosecretory granules were found close to the surface of the nerve. The motor axons were more centrally located. Figure 3a shows a neurosecretory axon near the surface of the proctodaeal nerve. In this figure, two adjacent axons are shown, one containing the characteristic neurotubules and the other showing a large cluster of neurosecretory granules. The diameter of these granules ranged from 1300 Å to 3900 Å.

From our earlier experiments, the site of action for the excitatory peptide seemed to be the visceral muscles of the hindgut. However, there was the possibility of a localized neural network which, if sensitive to the peptide, might indirectly stimulate the muscle fibers. This possibility was eliminated by treating an innervated hindgut with tetrodotoxin (10^{-6} g/ml). Four minutes after treatment, all neural function was abolished. At this time, the excitatory peptide was added to the muscle chamber, and the hindgut responded by showing all four characteristic features on the myogram (Fig. 4a). Thus, the excitatory peptide acts on the muscle fiber membrane rather than on the nerves leading to these muscles.

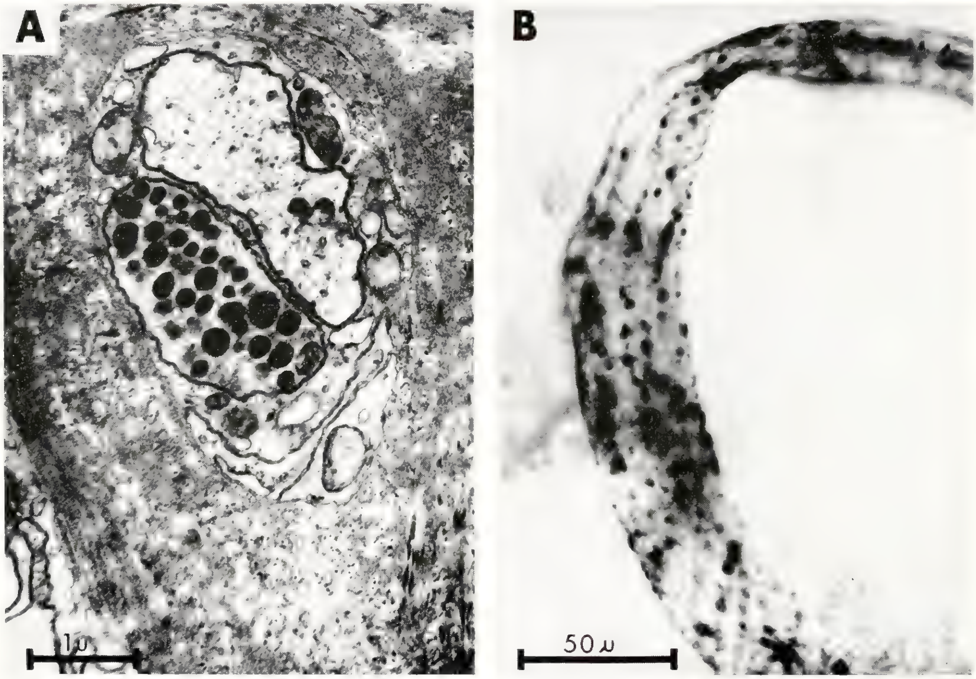

FIGURE 3. Neurosecretion in the proctodaeal nerve; (A) cross-section of 2 axons near the surface of the nerve showing neurosecretory granules in one and neurotubules in the other; (B) Victoria Blue staining of the proctodaeal nerve on the rectum.

Since Brown (1965) found that extracts of the corpora cardiaca had an excitatory effect on the hindgut as well as the heart of the cockroach, we decided to study what effect our peptide might have on an isolated heart preparation. Figures 4b and 4c show the response of the heart to the extracted peptide. In Figure 4b, 0.3 μg of dry residue was added to the heart at the point indicated by the arrow. A noticeable increase in frequency, amplitude, and coordination was evident. In Figure 4c, 1.0 μg of dry residue was added to the heart preparation, as indicated on the chart. At this higher concentration, heart activity was markedly depressed.

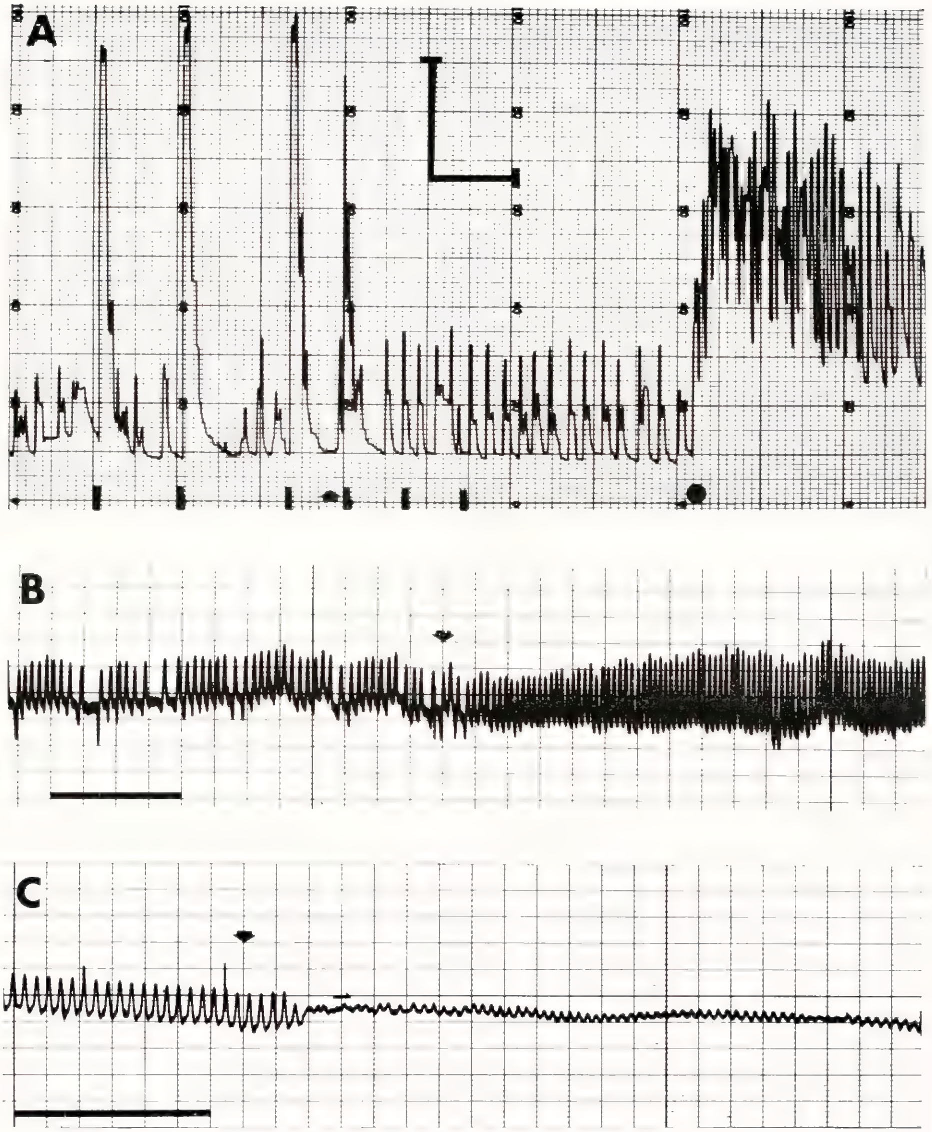

FIGURE 4. The response of tetrodotoxin treated hindgut and innervated heart to the excitatory peptide. (A) The proctodaeal nerves of the hindgut were stimulated at a pulse strength of 2 V, a duration of 1 ms, a frequency of 40 c/s, and a 2-sec train, as indicated by the vertical lines on the chart. The arrow marks the addition of tetrodotoxin (10^{-6} g/ml). The dot marks the addition of the peptide (3.2 μg of residue/ml time mark, 2 min; vertical calibration, 2 mm); (B) heart response to 0.3 μg of dry residue (1.2 μg/ml) of the extracted peptide (arrow); (C) heart response to 1.0 μg of dry residue (4 μg/ml) of the extracted peptide (arrow); time mark 40 sec. The heart was immersed in 250 μl of saline solution.

Discussion

Several investigators have extracted biologically active compounds from both the head and hindgut of the cockroach. Browne *et al.* (1961) extracted a compound(s) from *P. americana* hindgut and blood that induced contraction in the isolated rat uterus. This material was heat-labile and lost its activity when heated above 50° C for 10 min. Brown (1965), using paper chromatography, obtained two materials from extracts of the cockroach corpora cardiaca which stimulated hindgut myogenic activity. He designated these P_1 (also a stimulator of the heart) and P_2. Both materials were heat-stable and dialyzable. The dialysis rate showed P_1 to have the larger molecular weight. On the basis of biological assay, our compound is similar to P_1, as both hindgut and heart are stimulated. Factor P_1 was chymotrypsin-sensitive. Such is not the case for our substance. However, Brown (1965) described a loss of P_1 activity following incubation with heat-denatured chymotrypsin. Thus, it is difficult to assess whether the inactivation of P_1 by chymotrypsin was the result of enzymatic degradation or a non-enzymatic affect such as adsorption. Incubation of our compound with pronase completely abolished biological activity in less than 30 min, whereas incubation with heat-denatured pronase had no effect. This pronase inactivation demonstrates the amide nature of our compound.

Column chromatography provided us with a convenient method for comparing active substances extracted from different sources. Extracts of cockroach heads (including the corpora cardiaca) were found to contain an excitatory compound from hindguts. Subsequent comparison of the two active residues by thin-layer chromatography indicated that the active components had identical R_f values, whether they were chromatographed separately or as a mixture. Even with whole body extracts and a much more elaborate purification system, we have not been able to show the presence of more than one active substance that has the characteristic sustained effect on hindgut myogenic activity (Holman and Cook, unpublished data).

Natalizi, Pansa, d'Ajello, Casaglia, Bettini, and Frontali (1970) separated six biologically active materials from extracts of cockroach corpora cardiaca. Although they did not investigate the effect of these materials on the isolated hindgut, they showed four of them to be stimulators of the cockroach heart. Two of the materials also stimulated the spontaneous firing of the cockroach central nervous system (1 and 2), but the other two materials stimulated only the heart (5 and 6). Our peptide did not stimulate the spontaneous firing of the isolated cockroach nerve cord. The latter two materials (5 and 6) were retained by a carboxymethyl cation-exchange column, as was our compound. However, differences in cation-exchangers and solvents used preclude a more direct comparison between substances 5 and 6 and our peptide.

Gel filtration experiments with G-10 and G-15 Sephadex indicate a molecular weight of 400 to 600 for our compound. However, this could be misleading, as a chromatographic effect can cause certain materials to elute as lower molecular weight compounds. In fact, Natalizi *et al.* (1970) found this to be the case with all six biologically active components mentioned above. In their case, the effect was obvious, as the six materials were eluted after the salt fraction. However, their elution solvent differed from the one we used; again, direct comparison was not possible.

In summary, our evidence shows that the material we have isolated is a small, basic peptide.

Our preliminary survey of five insect species representing three orders shows that the peptide is not universally present. Thus far, it has been isolated only from representatives of the order Orthoptera. It was not present in the house fly (Diptera) or fifth-instar larvae of the tobacco hornworm (Lepidoptera).

The isolated hindgut of the American cockroach seemed to be just as responsive to the extracted peptide (regardless of the active source) as the hindgut from the Madeira cockroach. However, the isolated hindgut of the fifth-instar larvae of the tobacco hornworm showed no response to the peptide, even at several hindgut equivalents per ml.

Distribution studies of *L. maderae* showed that the hindgut contains the largest quantity of this peptide. It was also present in the head, terminal ganglion, and cercal-proctodaeal nerves. The peptide was neither present in the foregut nor did it affect the myogenic activity of that organ.

The ion-exchange chromatography of hindgut extracts resulted in a 2000-fold purification of the peptide (7.3 mg dry weight/hindgut *vs.* 3.2 μg residue/hindgut). A concentration of 0.004 hindgut equivalents/ml of this residue gave a threshold response (Fig. 1a). This corresponds to 13 ng residue/ml (Table I). The sensitivity to the pure compound is certainly much greater than this, as thin-layer chromatography of this residue showed that an estimated 90 per cent of the ninhydrin-positive materials were not associated with the R_f of the active compound.

Our extracted peptide seems to act directly on the membrane of muscle fibers. Not only was the hindgut still responsive to the peptide after treatment with tetrodotoxin, but both the isolated rectum and the colon reacted to the peptide. Thus, Davey's (1962) hypothesis of indirect stimulation of the gut through a peripheral neural network does not seem likely in the Maderia cockroach. Even in the American cockroach, a re-investigation of the neural relationships of the proctodaeal muscles (Brown and Nagai, 1969) did not reveal a peripheral neural network. However, a netlike syncytium of fibers was found which stained readily with methylene blue and had the structural characteristics of modified muscle.

Furthermore, the possible mediation of an indolalkylamine in the action of corpora cardiaca extracts on the hindgut of the cockroach as proposed by Davey seems remote. Both Colhoun (1967) and Brown quoted by Colhoun (1967) showed that bromolysergic acid diethylamine, a proven antagonist of 5-hydroxytryptamine and other indolalkylamines, failed to suppress hindgut responses to the addition of copora cardiaca extracts. Further, Davey (1962) remarked that the granules in the argentaffin-positive cells of the upper colon were positioned along the lumen side of the cell and would need some means of conduction to the muscle fibers above.

From our preliminary experiments on the innervated heart preparations, it was not possible to state whether the peptide affected the myocardium directly or indirectly via the pericardial cells, as proposed by Davey (1961). Furthermore, the lateral cardiac nerve cord may also have been affected by the peptide. In spite of these shortcomings, both the heart and hindgut showed similar response patterns to the peptide. Heart contraction frequency and amplitude increased, and the myocardiogram showed a more coordinated pattern (Fig. 4b). Surprisingly, at concentrations of 4.0 μg dry residue/ml, the heart displayed a marked inhibition which was reversible (Fig. 4c).

The presence of neurosecretory axons in the proctodaeal nerve of the Madeira cockroach was not entirely unexpected. Numerous instances of neurosecretory axons passing into or close to a variety of internal organs in insects have been reported. In aphids, neurosecretory tracts were found running to the hindgut and various muscles (Johnson, 1963). Both the rectal papillae of the blowfly (Gupta and Berridge, 1966) and the rectal pads of the cockroach (Oschman, cited by Maddrell, 1967) receive neurosecretory axons. In a situation where a synaptic-like termination is observed in contact with the target tissue, one might assume that the products of neurosecretion have a local action. In fact, Maddrell (1967, page 108) concluded . . . "that by no means all neurosecretory axons release horomones into the general blood system. Indeed it can only be confidently stated that the hormones are in fact normally released into the general blood system in relatively few cases. Even in these cases the evidence is often indirect." In the American cockroach, there are only two hormones for which there is direct evidence of this: the diuretic hormone (Mills, 1967) and the tanning hormone bursicon (Mills, 1966).

In conclusion, there are several facts suggesting that the peptide we have isolated is a neurohormone: (1) the peptide is present in extracts of the terminal ganglion and the cercal-proctodaeal nerve complex (Table I); (2) not only did the proctodaeal nerve stain selectively with Victoria Blue, but neurosecretory granules were evident in a number of axons appearing on electron micrographs of the nerve in cross-section (Fig. 3a, b): (3) although we have no direct evidence of the presence of the peptide in neurosecretory granules, preliminary centrifugation experiments in a sucrose medium showed that 97 per cent of the peptide is present in the particulate fraction at 20,000 *g;* (4) the sustained response of the visceral muscles of the hindgut and the heart to the peptide is in striking contrast to the very short duration of the postsynaptic responses encountered with neurotransmitter (Scharrer, 1969 and Holman and Cook, 1970); and (5) neurohormones are generally peptides (*e.g.*, vasopressin and oxytocin) which often act on diverse target organs. In the present instance, the same peptide has been found in both the head and the terminal ganglion, and it has the ability to alter the contractile properties of the heart and the hindgut but not the foregut.

Finally, although the precise location of release for the peptide in the hindgut is not known, the selective staining of the proctodaeal nerve and its branches with Victoria Blue over the entire hindgut certainly suggests a localized release. If indeed this proves to be the case, the neurosecretory substance would be more like a neurotransmitter than a typical neurohormone, at least from a histological point of view. Nevertheless, the persistent physiological effects of the peptide on the hindgut, even in submicrogram amounts, seem to necessitate describing it as either a local neurohormone (Burn, 1950 and Scharrer, 1969) or neuromodulator (Florey, 1967) which in some manner regulates the excitability of visceral muscle.

We are indebted to Mr. John Reinecke of this laboratory for the electron microscopy of the proctodaeal nerve. We also wish to acknowledge the competent technical assistance of Mrs. Patricia Kramer.

SUMMARY

1. Ethanol extracts of hindguts from *L. maderae* contained a material that stimulated the myogenic activity of the isolated organ. Ion-exchange chromatography of the ethanol extracts resulted in a 2000-fold purification of the active residue, and thin-layer chromatography demonstrated that the activity was associated with a single ninhydrin-positive spot.

2. The biological activity of the extracted substance was not affected by incubation for 3 hours with chymotrypsin. However, pronase completely destroyed the activity within 30 minutes, which suggests that the active material is a peptide. Gel filtration experiments indicated that the molecular weight is in the 400–600 range.

3. The peptide was isolated from extracts of terminal ganglia, proctodaeal nerves, and heads of the cockroach but not from the foregut. Interestingly, the myogenic activity of this organ was not affected by exposure to extracts from the hindgut. The peptide was found in the two cockroaches, *L. maderae* and *P. americana,* and the grasshopper, *S. vaga.* It was not found in the head of the house fly, *M. domestica* nor in fifth-instar larvae of the tobacco hornworm, *M. sexta.*

4. The characteristic response of the hindgut to this peptide was a sustained increase in the tonus, frequency, and amplitude of contractions. An excitation threshold was obtained with only 0.004 hindgut equivalents per milliliter or 13 ng of dry residue per milliliter. Both the isolated rectum and colon responded to the peptide, and although tetrodotoxin (10^{-6} g/ml) blocked neurally evoked contractions, the visceral muscle of the hindgut showed no change in sensitivity to the extracted peptide.

5. Cockroach innervated heart preparations showed a noticeable increase in frequency, amplitude, and coordination of contractile events when exposed to 0.3 μg of dry residue. If 1.0 μg or more of the dry residue was added to the heart, activity was greatly depressed.

6. Both histological and cytological evidence of neurosecretion were obtained from the proctodaeal nerve. Preliminary centrifugation experiments suggest that the extracted peptide is associated with the particulate fraction at 20,000 *g*. Finally, a number of reasons for considering our extracted peptide as a neurohormone are discussed.

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THE ACTION SPECTRUM OF LIGHT INDUCED AGGREGATION IN *POLYSPHONDYLIUM PALLIDUM,* AND A PROPOSED GENERAL MECHANISM FOR LIGHT RESPONSE IN THE CELLULAR SLIME MOLDS

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For a long time it has been known that light has a stimulatory effect of aggregation in the cellular slime molds. The response has been recorded variously by different authors as an effect on the time of aggregation, the number of aggregates, or the size of the aggregates, and it has not been clear whether there is a single primary response common to these differing effects. The picture is further confused by the fact that the details of the response are different from one species to another.

The work that we report here is intended to carry one step further the understanding of the light response in one species, *Polysphondylium pallidum,* by providing an action spectrum for the response. In addition, we propose a general mechanism which may be responsible for the various forms of the light response.

MATERIALS AND METHODS

We used *Polysphondylium pallidum,* strain WS 320, kindly supplied by K. B. Raper. Stock cultures were kept on *Escherichia coli,* strain 281, on agar made with 0.2% Cerophyll. (Cerophyll consists of powdered cereal grass leaves and is available from Cerophyll Laboratories, Kansas City, Missouri.) Amebae for experiments were grown axenically by inoculating spores from the stock plates into the following medium, modified from Sussman (1963):

Lecithin (Eastman Kodak, practical grade)	−0.4 g
Proteose peptone (Difco)	−10 g
Glucose	−1 g
$Na_2HPO_4 \cdot 7H_2O$	−0.72 g
KH_2PO_4	−1.45 g
Streptomycin sulfate	−10 mg
Dist H_2O	−1 liter

The flasks were incubated on a shaker at 23–26° C, in constant fluorescent light of ~50 foot candles intensity. After 3–4 days the amebae were harvested by centrifugation, washed once in cold Bonner's salt solution (Bonner, 1947) and dispensed as 10 λ drops onto plates of 1.5% Difco purified agar made with distilled water, which had been poured 2–3 days in advance. This procedure of plating was initiated by Konijn and Raper (1961). The drop soon dries, leaving a population of amebae which remain within a small area on the agar surface. After plating the dishes were incubated in constant darkness at 21 ± 1° C.

At a time and in a manner to be detailed in the results section, the plates were exposed to light for a brief period then returned to darkness.

As light sources we used either a General Electric cool white fluorescent lamp at 4.1×10^3 ergs/cm^2/sec intensity, or monochromatic light provided by a microscope lamp in combination with Schott interference line filters. Approximately 99% of the energy transmitted is within a 20 millimicron interval with these filters. The brightness of the lamp was controlled by a Variac transformer, and a four-inch water bath was interposed in the light beam to absorb infrared energy. The intensity was measured with a YS1-Kettering model 65 radiometer and a Photovolt Corporation model 520M photometer. The photometer was necessary for measuring the lower intensities and was calibrated (in terms of ergs/cm^2/sec) at each wavelength by using the radiometer.

Six hours after light exposure the plates were taken from the incubator and the number of aggregation centers per drop was recorded.

TABLE I

Effect of length and time of exposure on density of aggregation

	Length of exposure (min)			
	0 (Background)	1/4	2	30
Time at which exposed (Hours after plating)				
1	26 centers/drop	26	26	32
5	24	33	46	43
17–20	32	43	40	44

RESULTS

Response to brief periods of illumination

Our first experiment was designed to select an appropriate time and length of exposure for later use in determining the action spectrum.

The amebae were plated and incubated as described above, then exposed to fluorescent light for varying brief periods, then returned to the dark and later the aggregates counted. The results of one typical experiment are shown in Table I and may be summarized as follows:

(a) It is clear that the background number of aggregates is rather high: 24–32 centers per drop appear without any light exposure. This is in contrast to Kahn's (1964) results with a similar experiment where the background was near zero. The background appears to increase slightly from 5 to 17–20 hours, and repeated tests show that this is generally true (Fig. 1).

(b) In the two cases where the number of aggregates in exposed plates is clearly above background, *i.e.*, at 5 and 17–20 hours, a two-minute exposure is as effective in inducing aggregates as a 30-minute exposure. Kahn (1964) found a similar result.

(c) The time at which exposure is most effective in increasing the number of aggregates is at 5 hours after plating. The total number of aggregates per drop is essentially the same at 17 hours, but the background is higher in that case. Exposure at one hour after plating has almost no effect. This result is in apparent conflict with Kahn's (1964) conclusion that the sensitivity to induction decreased from 1 hour to 6 hours. A possible cause of the disagreement is that Kahn used amebae grown in darkness, whereas ours were light grown; for it seems reasonable that amebae which have been taken from constant light should be insensitive to a brief flash given only one hour later. Also, growth in light might cause induction of some center forming cells before plating, and so might explain our high background.

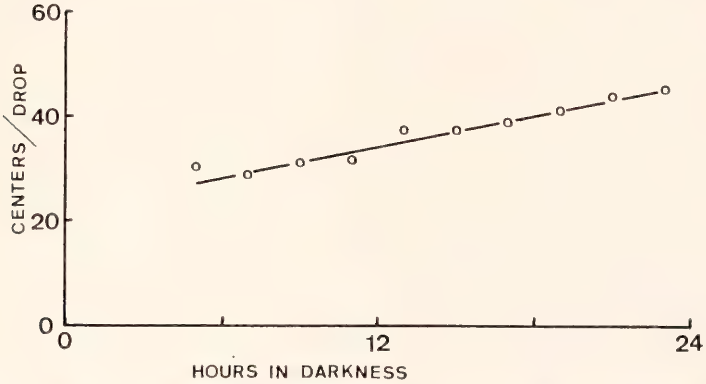

FIGURE 1. Increase in number of aggregations in cell populations kept in constant darkness. The drops were plated at T = 0. Points are averages of counts made on 6 drops. Culture harvested at 4.0×10^4 cells/ml, plated at 5.0×10^5 cells/ml.

Action spectrum

We concluded that 2 minutes of light given at 5 hours after plating would be a suitable exposure to use in determining an action spectrum and that intensities around 4×10^3 ergs/cm^2/sec would be relevant. The experiments were performed in the same way as above, except that the amebae were exposed to monochromatic light instead of white light. The same intensity was used at each wavelength, so as to give an *equal energy* action spectrum. The results of three separate experiments at two intensities are shown in Figure 2. There are two main peaks of activity, one at 475 mμ, one at 675 mμ.

DISCUSSION

The many workers who have studied the light response of cellular slime molds have recorded effects on a variety of processes. The more important of these are the following:

(*a*) *Effect on the time of aggregation*

Most experimental arrangements have used cells plated on agar in the absence of food. After plating a certain time elapses before aggregation begins, and this time is one phenomenon which is influenced by light. Konijn and Raper (1965) found that aggregation occurs soonest in constant light in *P. pallidum*, which in this respect differs from *D. discoideum* and *A. rosea* where a dawn following a night of certain length is optimal (Konijn and Raper, 1965; Reinhardt, 1968). In all of these cases, light has its effect on post-vegetative, preaggregative cells, and must induce in them some cellular process which leads to aggregation. Some observations by Shaffer (1961) give a hint as to what this process might be. He found that aggregations in *P. violaceum* are started by special founder cells which apparently secrete large amounts of acrasin, for they directly attract neighboring cells. Furthermore, Shaffer noted that the number of founders increased sud-

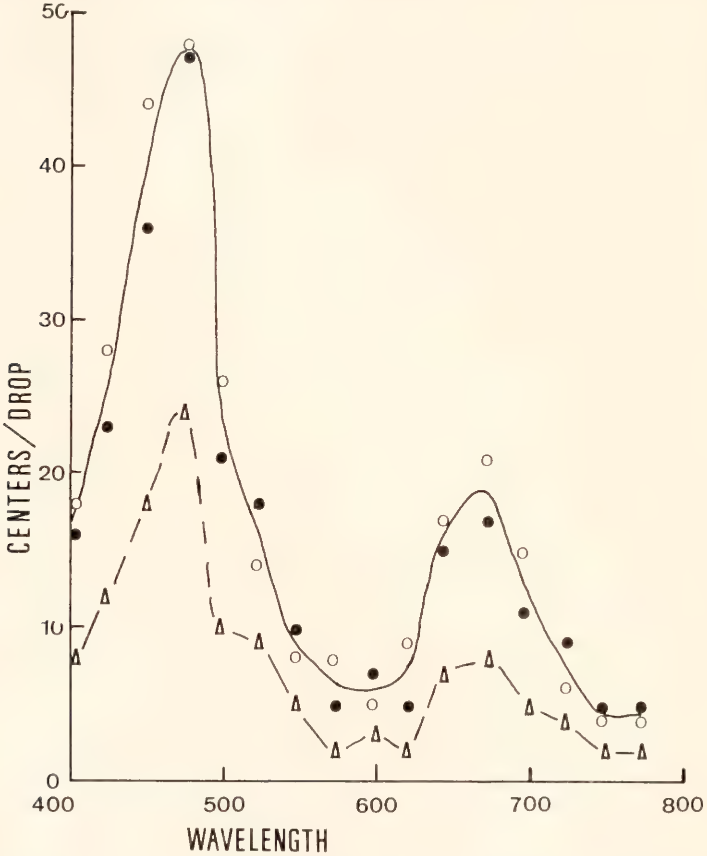

FIGURE 2. The action spectrum of light induced aggregation. Centers/drop above background (ordinate) are plotted against wavelength in mμ (abscissa). "Background" is the number of centers which appear in drops kept in constant darkness. Each point is the average of counts made on 6 drops. ○, ● indicate results of two separate experiments using 9.2×10^3 ergs/cm^2/sec at each wavelength. Cultures were harvested at 1.2×10^6 cells/ml, and plated at 5×10^5 cells/ml; background equals 45 and 50 centers/drop. △ indicates results of a third experiment using 4.8×10^3 ergs/cm^2/sec at each wavelength. Cultures were harvested at 1.5×10^6 cells/ml, and plated at 1.0×10^6 cells/ml; background equals 48 centers/drop.

denly following a flash of light. This suggests that light can induce the production of acrasin in at least some cells. Founder cells also occur in *P. pallidum* (Francis, 1965), and quite probably their appearance is stimulated by light in this species as well, although no direct observations on this point have been made.

(*b*) *Effect on the number of centers per unit area*

In all species which have been studied the number of aggregations per unit area is higher when the population has been exposed to light (*D. discoideum*—Konijn and Raper, 1966; *P. pallidum*—Kahn, 1964; *A. rosea*—Reinhardt and Mancinelli, 1968). Where light flashes have been employed to investigate this phenomenon it is clear that one sensitive period is the same preaggregative phase mentioned above which is several hours before macroscopic aggregations appear. Perhaps this means that the primary event here is also the induction of founder cells.

(*c*) *Effect on the size of aggregates*

This measure is partly correlated with the effect on number of centers, since if there are more centers per unit area (and if all of the amebae enter some center) each center must necessarily be smaller. Especially when this criterion of light stimulation is used it becomes clear that light has an effect not only on preaggregative cells, but also on the later stages of aggregation. For example, Reinhardt (1968) has noted that in *A. rosea,* light causes the breaking up of large old aggregates. Because of this second period of sensitivity to light it is important to note the time after initial exposure to continuous light at which aggregates are counted. The number of induced aggregates may be quite different when counted at 6 hours after initial exposure when aggregates are just becoming macroscopically visible, and when counted at 24 hours when the first formed aggregates have possibly been dissolved and new ones have formed. So as to make doubly sure of avoiding these secondary effects we counted aggregates at 6 hours, even though in our experiments the developing aggregates were kept in darkness after the brief exposure.

(*d*) *Effect on orientation of the pseudoplasmodium*

In several species the migrating or stalk-forming pseudoplasmodium orients toward a source of light. The effect occurs at the tip of the body and on the side away from the light source, and may possibly be an increase in the motive power of the amebae in this zone (Francis, 1964).

In view of such variety in the expression of response to light and in the diversity of the behavior of different species, one may wonder if there can in fact be a common mechanism of reaction. Nevertheless we want to put forward a possible basic mechanism, which is suggested by an analogy of the phenomena of aggregation with the processes of light reception in the metazoan eye. As summarized by Bitensky, Gorman and Miller (1971), the response in the vertebrate retina consists of light trapping by the pigment 11-cis retinal, followed by hyperpolarization of the retinal cell membrane. In invertebrate eyes the linkage is in the opposite direction and light causes *de*polarization (Miller, Gorman and Bitensky, 1971). Bitensky showed that the intermediate process between light reception and hyper- or depolarization involves a change in activity of adenyl cyclase. It would seem that this is followed by a corresponding change in concentration of cyclic-AMP, which then in some way alters the permeability of the membrane to Na^+, resulting in the change in membrane potential.

These events may be compared with what happens during aggregation in the cellular slime mold. Here Bonner's group (Bonner, Barkley, Hall, Konijn, Mason, Keefe and Wolfe, 1969) has recently shown that a key event is the sudden increase in cyclic-AMP, at least in *D. discoideum.* Cyclic-AMP is one naturally occurring acrasin, and attracts cells to the forming centers. How the chemotaxis operates is not yet completely understood, but it is known that cyclic-Amp can change the membrane permeability to Ca^{++} (but not Na^+) in the cells of *D. discoideum* (Chi and Francis, 1971), and that Ca^{++} activates the ATP-ase activity of contractile proteins in amebae of cellular slime molds (Woolley, 1970). These known similarities between the retina and aggregation suggest that the two systems might be similar in other aspects as well. In particular, it seems useful to hypothesize that the unique immediate effect of light on the cellular slime molds is to induce production of cyclic-AMP or acrasin. The suggested analogy is outlined in Table II.

TABLE II

Proposed parallels between the processes of aggregation and vision occurring after light stimulation

	Retina	Aggregation
1	Light + 11-cis retinal	Light + unknown pigment
2	Change in C AMP production by adenyl cyclase activation, or inactivation	Changed production and release of C AMP or other acrasin by attracting cells
3	Change in Na^+ permeability and membrane potential of retinal cell	Local change in Ca^{++} permeability and intracellular Ca^{++} in responding cells
4	Impulse in optic nerve	Chemotaxis of responding cell, caused by assymetric operation of contractile system

Some of the light stimulated reactions of cellular slime molds already listed are explicable by an increased acrasin production in some or all cells. The increase of visible founder cells following light exposure is one of these, since the easiest interpretation of a founder cell is that it is a cell secreting exceptional quantities of acrasin. Early or more frequent induction of founder cells will lead to faster and more numerous aggregates, as mentioned earlier, and explain the light stimulation of aggregation. The breaking up of already formed centers is not so obviously derived from increased acrasin production, although it is conceivable that the chemotactic gradient surrounding an aggregate might be disrupted if many of the peripheral cells suddenly begin to produce large amounts of acrasin. This could explain the case of *A. rosea.* Further, Konijn (personal communication) has noted that a very high concentration of C AMP in the agar substrate brings about disintegration of previously existing centers. Phototaxis is a phenomenon which might easily be caused by a chain reaction leading from light stimulation through acrasin production to altered cell movement, as outlined above. It should be carefully noted that we here used cyclic-AMP and acrasin interchangeably. In actual fact, of course, cyclic-AMP is the acrasin for only some of the *Dictyostelium* species but not for *Polysphondylium* (Bonner *et al.*

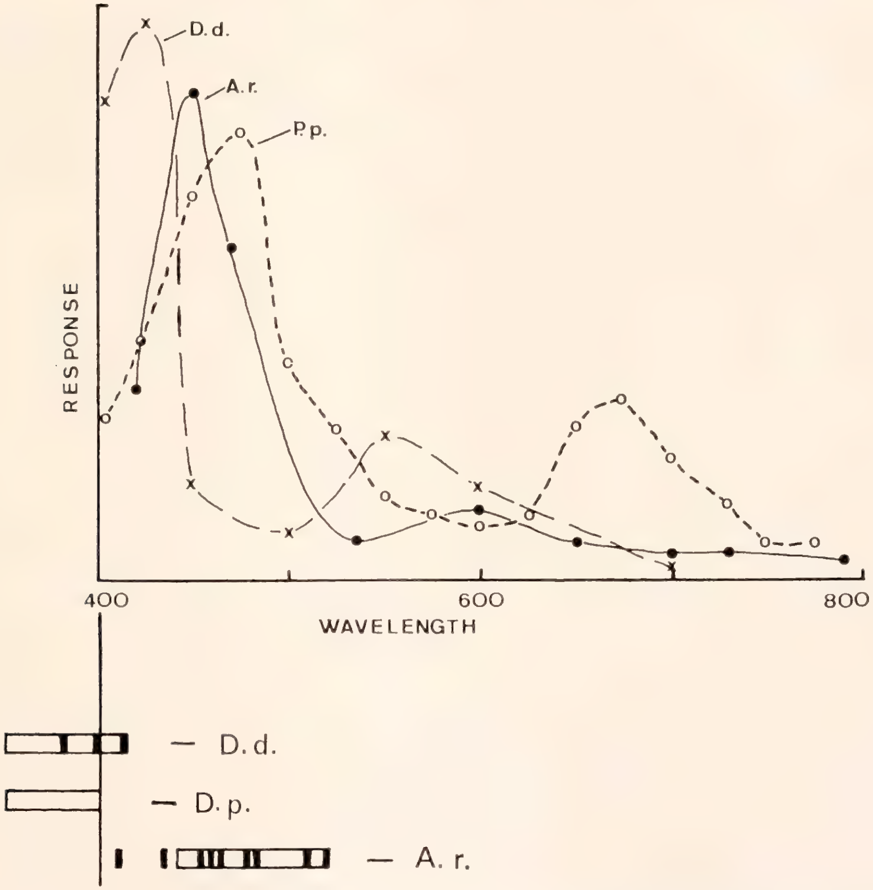

FIGURE 3. Comparison of action spectra (above) and absorption spectra (below) for three different species. For action spectra the units of response are different in each case. D.d. indicates phototaxis of *D. discoideum* and *D. purpureum* (Francis, 1964). The response is the reciprocal of the amount of energy in ergs/cm^2/sec necessary to produce a standard phototactic turn. A.r. indicates induction of aggregation in *A. rosea* (Reinhardt and Mancinelli, 1968). The response is the number of centers per standard drop of amebae. Equal intensities were not used at all wavelengths, but the intensity at 450 mμ was weaker than the rest so that this peak is real. P.p. indicates induction of aggregation in *P. pallidum*, from Figure 2. For absorption spectra the boxes indicate the regions of absorption, bars indicate peaks in absorption. Abbreviations are D.d, ethanol extract of carotenoids of *D. discoideum* (Staples and Gregg, 1967); D.p., cell-free liquid from spore heads of *D. purpureum* (Francis, 1964); A.r., hexane solutions of several carotenoids of *A. rosea* (Fuller and Rakatansky, 1966).

1969), and it is an additional assumption that in *Polysphondylium* light has the same effect on production of the unknown acrasin as it is postulated to have on production of cyclic-AMP in *Dictyostelium*. The main value of the hypothesis is that it provides a mirror within which these several phenomena can be viewed from a new angle. We hope that it will be of use in suggesting new experiments.

Action spectra have been determined for light reactions of two other species of cellular slime molds. In these cases the responses were the induction of aggregation in *A. rosea* (Reinhardt and Mancinelli, 1968) and the phototaxis of *D. discoideum* and *D. purpureum* (Francis, 1964). The three spectra are by no means identical, as may be seen in Figure 3, although all show a major peak in the 424–475 mμ region. Figure 3 also shows several absorption spectra of pigments extracted from cellular slime molds. Staples and Gregg (1967) have suggested that the carotenoid which they isolated from *D. discoideum* may be responsible for the 425 mμ peak in the action spectrum of phototaxis for this species. Similarly, the carotenoids extracted from *A. rosea* by Fuller and Rakatansky (1966) would seem likely receptor pigments for the action spectrum of the same species. Whether carotenoids are in fact necessary for these light mediated responses cannot be decided from mere coincidence of peaks in action and absorption spectra, however. More crucial experiments would be to show that the response is removed when carotene synthesis is depressed by chemical inhibitors (Staples and Gregg, 1967) and to show that nonphototactic mutants (Loomis, 1970) lack carotenoids.

After these experiments had been completed we learned of the work of Kientzler and Zetsche (1972), who have also determined an action spectrum for induction of aggregation in *Polysphondylium pallidum*, under somewhat different conditions from ours. They found peaks at 460 and 600 mμ, which shifted to 450 and 550, respectively, when the intensity and length of exposure were increased. It is difficult to understand why the spectral shift occurs, and why both spectra differ from the one that we found.

None of the spectra resemble the action spectra of the metazoan visual response which has a maxium of 375 mμ and another at 500 mμ when the pigment is protein-conjugated (Wald, 1968). Nor is it known whether the carotenoids of cellular slime molds are normally associated with proteins. We emphasize that the comparison of the metazoan visual system and cellular slime mold aggregation is best taken at present as an analogy in which the relations between certain processes like light, cyclic AMP, and permeability to a specific ion, may be similar, although details such as the nature of the pigment and the particular ion to which the membrane becomes permeable are different in the two cases. As yet we have much too little evidence to suggest any true evolutionary homology of the two systems.

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SUMMARY

In the cellular slime mold, *Polysphondylium pallidum*, aggregations are induced by a brief period of light coming after a long (5 hour) dark period. The

action spectrum of this response was determined by illuminating small populations of amebae with a constant sub-saturation amount of light at each of several different wavelengths and measuring the number of aggregates induced in each case. Peaks in response occurred at 475 and 675 mμ. One of these peaks falls on the absorption maximum of hexane extracted carotenoids from another cellular slime mold, *Acrasis rosea,* suggesting that the receptor pigment may be a carotenoid.

The light responses of the cellular slime molds are briefly reviewed. It is suggested that the events during light stimulation of aggregation and during other light induced responses may be similar to those which occur during light stimulation of the metazoan retina. In that case, the initial cellular even common to all light responses of the cellular slime molds may be the stimulation of cyclic-AMP production.

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A NEW SPECIES OF MARINE LEECH (ANNELIDA: HIRUDINEA) FROM SOUTH CAROLINA, PARASITIC ON THE ATLANTIC MENHADEN, *BREVOORTIA TYRANNUS*[1]

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The current systematics of marine leeches is in a confusing state in spite of recent attempts to review the group (Knight-Jones, 1961; Soós, 1965). Unfortunately, the internal anatomy of many of the type species has been so inadequately described that in most cases the exact limitations of the genera are only vaguely known. Recent workers have pointed out also that an initial dichotomy based on environment (*i.e.*, freshwater or marine) is completely unsatisfactory (Hoffman, 1964). In contrast to the freshwater piscicolid leeches of the United States and Canada which have been thoroughly investigated by Meyer (1940, 1946a, 1946b), very little is known of American marine piscicolids. Although at least eight marine fish leeches have been reported along the eastern American coasts, a comprehensive morphological study is lacking and new species and even genera are not uncommonly encountered. Since 1963, we have sporadically encountered a previously undescribed marine piscicolid of the *Calliobdella-Cystobranchus-Piscicola* complex in the vicinity of Charleston, South Carolina. The mid-body segments of the young (3 mm) of the new species are made up of 3(6) annuli which, upon maturation, are further subdivided into the typical 7(14)-annulate condition of the adults. A similar subdivision of the mid-body annuli upon maturation was found in the marine leech *Oceanobdella blennii* by Sawyer (1970), who discussed the systematic significance of annulation in the piscicolids. In this paper we hope to contribute to the stabilization of the systematics of marine piscicolids by keeping with the modern approach of giving systematic emphasis to the more conservative internal structures, especially the reproductive systems, and minimizing the importance of such external characters as the degree of annulation, the emphasis on which in the past has led to a confusing proliferation of genera. A detailed morphological examination has shown the reproductive systems and other internal structures of the new species resemble so remarkably those of the genus *Calliobdella*, which has only two known species (Soós, 1965) that the new species must be assigned to this genus.

Family Piscicolidae, Johnston, 1865

Genus *Calliobdella* van Beneden and Hesse, 1863

Type species (by original designation): *C. lophii* van Beneden and Hesse, 1863.

Revised definition of genus: Body sub-cylindrical, not sharply divided into urosome and trachelosome; smooth without papillae or tubercles; caudal sucker wider

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[1] Contribution No. 22 of the Grice Marine Biological Laboratory.

than the maximum width of the body and about twice the width of the oral sucker; mouth central; 0–2 pairs of eyes; mid-body segments 3(6)- or 7(14)-annulate; six pairs of testes; 11–13 pairs of pulsatile vesicles; coelomic system well developed; posterior crop ceca fused, with fenestrae; esophageal diverticula and conducting tissue present; no external copulatory zone; with a medial, muscular organ associated with the bursa; marine.

Calliobdella carolinensis sp. nov.

Figures 1–3

Total length of sexually mature adults including suckers, about 18 mm (range, including juveniles 2.3–30 mm); first two nuchal annuli constricted; oral and caudal suckers eccentrically attached; mid-body segments 14-annulate; two pairs of cephalic ocelli; no ocelli on caudal sucker; transverse bands of metameric pigmentation on oral sucker and segments VII through XII; 11 pairs of pulsatile vesicles on segments XIII through XXIII; 12 pairs of metameric pigment spots from segments XIII through XXIV; anterior portion of vas deferens convoluted and extending to ganglion IX; a well-developed medial, muscular organ which functions as a seminal receptacle confluent anteriorly with the bursa and posteriorly with the ovisacs. Known hosts: Clupeidae Fish: the Atlantic menhaden *Brevoortia tyrannus* (Latrobe) and the blueback herring *Alosa aestivalis* (Mitchill).

TYPE LOCALITY: Beresford Creek near the Wando River, Berkeley County, about 13 km north-northeast of Charleston, South Carolina (32°53.2′N; 79°52.7′W). Host, *Brevoortia tyrannus* (Latrobe), captured by D. L. Hammond in an otter trawl near mud bottom in about 6 m of water. Bottom water temperature: 10.1° C; salinity: 13.0‰. In all, twelve leeches, ranging in length from 7.5 mm to 30.0 mm, were found on 12 January 1971 on *B. tyrannus* and on the deck of the boat. The holotype and three of the paratypes are deposited in the Grice Marine Biological Laboratory (Reference numbers: Holotype 71–138–1; Paratypes 71–138–2). The other paratypes are deposited in the Charleston Museum (Reference number: 71–67) and in the U. S. National Museum (Reference number: 45601).

HOLOTYPE (Fig. 1, A-F): The elongated body, translucent in the living animal, is barely perceptibly divided into a rounded trachelosome (length, 3.5 mm) and a more flattened urosome (length, 13.0 mm). Total length, inclusive of the suckers, is 18.5 mm; the width of the posterior sucker (2.0 mm) is greater than that of the body at its maximum width (1.6 mm), and twice the width of the oral sucker (1.0 mm). The ratio of the maximum body width to length is 1 : 11.6. The mouth is centrally located in the deeply cupped, almost hemispherical oral sucker, which is eccentrically attached to the neck immediately above its posterior edge. The first two or three nuchal annuli are so narrowly constricted (0.4 mm) that they can be telescoped slightly into the other annuli of the neck. The neck annuli gradually widen to become continuous with the externally unmarked clitellar (X through XII) region. From segment XIII the slight but perceptible widening of the body marks the beginning of the urosome which is slightly flattened dorsoventrally. From segments XIII through XX the sides of the body are almost parallel. Eleven well-defined pairs of lateral pulsatile vesicles occur immediately posterior to the ganglia in segments XIII-XXIII, inclusive. The vesicles become progressively smaller as well as positioned relatively more posteriorly in their respective segments. The surface of the body and the suckers is otherwise smooth, no papillae, tubercles or gills being present. Upon clearing with xylol, some of the internal organs can be detected: the tubular proboscis and large salivary glands extending posteriorly to segment IX, inclusive, coiled sperminiferous tubules almost as far anterior as IX, six pairs of testisacs in segments XIII through

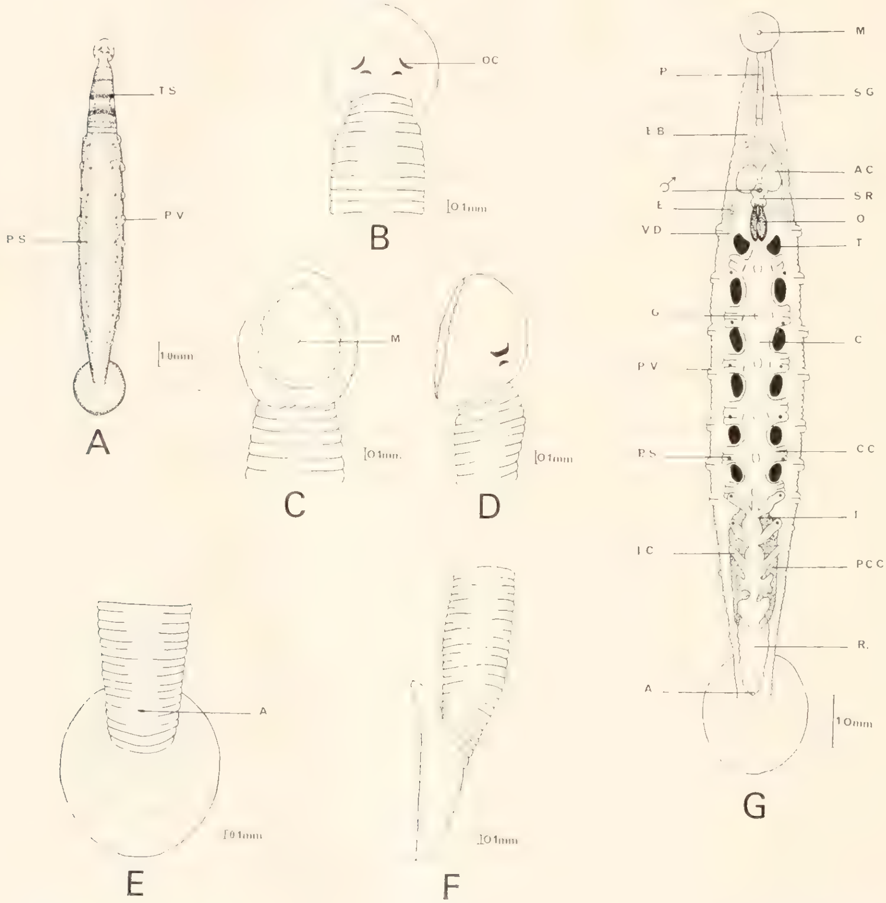

FIGURE. 1. *Calliobdella carolinensis* sp. nov.; A-F, holotype; A, dorsal view of entire individual; B-D, close-up of oral sucker; B, dorsal view; C, ventral view; D, lateral view; E-F, close-up of caudal sucker; E, dorsal view; F, lateral view; G, reconstruction of the digestive and reproductive systems; a, anus; a.c, atrial cornus; c, crop; c.c, crop cecum; e, epididymis; e.b, ejaculatory bulb; g, ganglion; i, intestine; i.c, intestinal cecum; m, mouth; o, ovisacs; oc, ocellus; p, proboscis; p.c.c, posterior crop cecum; p.s, pigment spot; p.v, pulsatile vesicle; r, rectum; s.g, salivary gland; s.r, seminal receptacle; t, testisac; t.s, transverse stripe; v.d, vas deferens.

XVIII, metameric crop and intestinal ceca, and numerous clitellar gland cells in the urosome. The mid-body segments appear 7(14)-annulate, but this is somewhat obscured. The annulation is reduced in the clitellar region, X through XII. The male gonopore is slightly elevated ventrally, but the female gonopore could not be found externally. The anus is barely visible about three annuli from the posterior sucker. The caudal sucker is somewhat longer (2.2 mm) than wide (2.0 mm), eccentrically attached to the body about one-third the distance from the anterior rim.

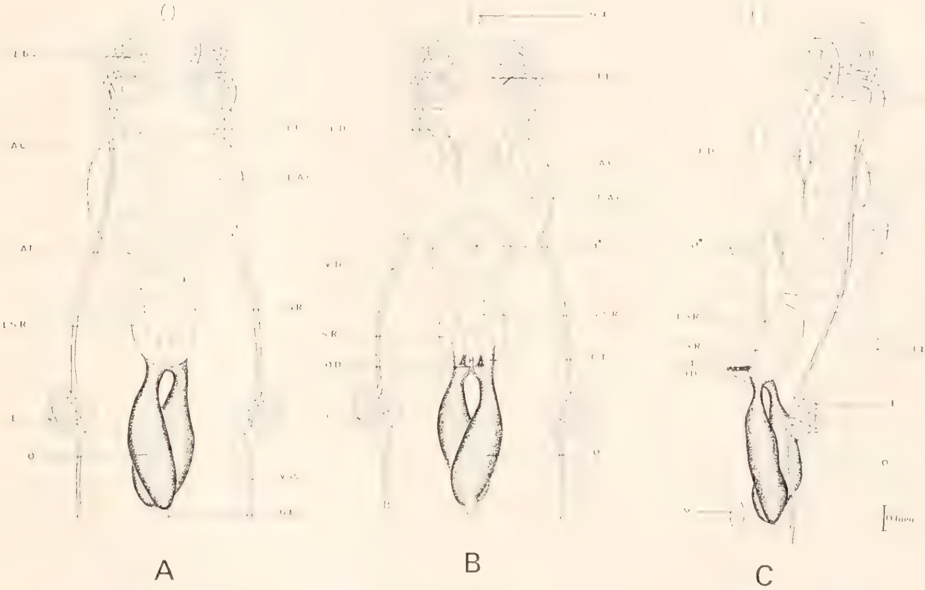

FIGURE 2. *Calliobdella carolinensis;* reconstruction of the reproductive systems. The dotted lines indicate the outlines of the lumina of the atrium, atrial cornua, bursa, and the seminal receptacle; A, dorsal view; B, ventral view; C, lateral view; a.c, atrial cornua; at, atrium; c.t, conducting tissue; e, epididymis; e.b, ejaculatory bulb; e.d, ejaculatory duct; g, ganglion; l.a.c, lumen of atrial cornua; l.at, lumen of atrium; l.s.r, lumen of seminal receptacle; o, ovisac (shaded); o.d, oviduct; s.r, seminal receptacle; v.d, vas deferens. (The female gonopore was not discernible externally.)

Two pairs of crescent-shaped ocelli, consisting of web-like concentrations of black pigment, are located on the anterior sucker (Fig. 1, B), a large anterior pair directed anteriorly and slightly laterally, and a small, more medial pair directed slightly posteriorly. No ocelli occur on the caudal sucker. Scattered over most of the suckers and body are two types of large chromatophores: a peripheral, reddish-brown and a deeper, blackish type. Faint indications of metameric pigmentation consist of narrow transverse bands, especially toward the anterior end (Fig. 1, A): one faint band on the oral sucker in the region of the posterior pair of ocelli, four distinct bands on the trachelosome, and three narrow bands in the clitellar region. These bands are not readily distinguishable on the urosome. Along the lateral margins of the urosome are thirteen faint pairs of triangular-shaped whitish areas devoid of chromatophores. Twelve pairs of pigment spots are barely discernible dorsally from segments XIII through XXIV (possibly a thirteenth in segment XXV), and eight pairs ventrally in segments XIII through XX. They are located near the body wall posterior to the ganglion and anterior to the vesicle.

PARATYPES AND VARIATIONS: More than 500 individuals of *C. carolinensis* have been collected and examined alive from several localities around and in Charleston harbor, ranging in size from 2.3–30 mm. They all possess two pairs of ocelli on the oral sucker, no ocelli on the caudal sucker, and the two types of scattered chromatophores. In addition, most of the living individuals display: twelve pairs of dorsal pigment spots on segments XIII through XXIV, faint indications of metameric pigmentation, six pairs of testisacs (visible with transmitted light), blood-filled crop ceca and intestinal ceca, a barely detectable division between urosome and trachelosome, 14 (sometimes 12) annuli per mid-body segment (sometimes obscure and hard to interpret), and 11 pairs of pulsating lateral vesicles. The annulation closely resembles that described for *Marsipobdella sacculata* by Moore (1952), except the testisacs do not extend so far posteriorly in the segment. There is a barely detectable metameric sequence in the furrowing of the annulation with the deepest furrow (probably at $C_{5,6}/C_7$) immediately anterior to the vesicle. Within the intestines of most individuals just in front of the last pair of vesicles is a conspicuous blackish ball, presumably partially decomposed food.

The basic body shape is often grossly distorted during fixation and the faint indications of metameric pigmentation is often obliterated after being in preservative for some time. If preserved without prior relaxation with weak ethanol the pulsatile vesicles are often obliterated, and even after careful preservation only six or seven vesicles may remain intact. Similarly the twelve pairs of urosomal pigment spots, never very conspicuous in the living animal, may be lost or show up only upon clearing. Only six or seven pairs of spots may remain intact. The caudal sucker can be folded along the longitudinal axis. The tertiary nature of the mid-body annulation is especially affected by poor preservation, so that at times the segments appear to have 6(7) annuli per segment. If preserved without prior relaxation, the male bursa may be everted posteriorly and the proboscis may be everted through the mouth. The smaller, immature individuals (2–10 mm) differed from the adults in being much less pigmented, more translucent, almost cylindrical with less of a division between trachelosome and urosome, and less conspicuous or even internal pulsatile vesicles. Similar differences between young and adult marine piscioloids were noted in *Oceanobdella blennii* by Sawyer (1970).

This section is based on the histological examination of four representative individuals, 3, 14.6, 17, and 18 mm in length, all sectioned at 15–20 mu and stained with Ehrlich's haematoxylin and eosin. The leech nervous system consists of a chain of thirty-four ganglia connected by a double nerve cord. The first six (I–VI) are aggregated to form the brain and the last seven (XXVIII–XXXIV) are aggregated to form the caudal mass. The remaining twenty-one ganglia (VII–XXVII) are segmentally arranged between them.

COELOMIC SYSTEM: The well-developed coelomic system resembles that of *Calliobdella, Cystobranchus, Piscicola,* and *Trachelobdella* (Type I of Selensky 1915).

The ventral sinus, which surrounds the nerve cord and the ventral blood vessel, is especially prominent in the region of the ganglia where wing-like projections extend dorso-laterally, but is reduced in the testicular region. The dorsal sinus, which surrounds the dorsal blood vessel, is especially prominent in the interganglionic regions. Both the ventral sinus and the dorsal sinus extend most of the length of the body. Small sinuses are also associated with the ovisacs and testisacs. A large sinus surrounds most of the intestine. The paired lateral sinus, which extends from about ganglion XIII to the intestinal region, is more prominent in the interganglionic regions, especially along the anterior portion of its length. It is continuous with the dorso-lateral projections from the ventral sinus as well as with a dorsal transverse sinus, which traces a somewhat tortuous path from the lateral sinus anteriorly and medially to the dorsal sinus.

Within each pulsatile vesicle there is a two-chambered sinus lined with a flattened epithelium and located outside the body wall. From the posterio-dorsal portion of the dorsal chamber extends a tubular sinus connective through the body wall. It has not been determined whether this connective is continuous with the lateral sinus. The coelomic system of the immature (3 mm) specimen is somewhat reduced. The pulsatile vesicles especially differ from the adult condition in being quite small and internal.

Digestive system (Fig. 1, G): The proboscis, a tube 125 μ in diameter with a narrow triangular lumen, extends posteriorly to segment IX. The paired salivary glands, up to 140 μ in length, are located between ganglia VII and IX. The esophagus enlarges laterally immediately posterior to the proboscis. A pair of anteriorly directed lateral ceca from the esophagus is confluent with the gut at ganglion XI, but it has not yet been determined whether these correspond with the esophageal diverticulae ("esophageal gland") located at this position in many marine piscicolids (see Sawyer, 1970). The gut lumen narrows somewhat in segment XI and expands laterally into the crop at segment XII. The crop lumen narrows in the testicular regions of segments XIII to XVIII, inclusive, and expands laterally as crop ceca in the intertesticular regions of these segments. Well-developed dorso-ventral muscles occur on either side of the gut between the crop ceca. The intestine and the posterior crop ceca originate just posterior to ganglion XIX. The convoluted intestine has five pairs of anteriorly directed, convoluted ceca. The posterior crop ceca, which extend from segments XIX to XXV), inclusive, are fused except for a fenestra in each segment through which dorso-ventral muscles or strands of connective tissue pass.

Reproductive system (Fig. 2, A–C; Fig. 3, A–F): The six pairs of testisacs, immediately posterior to the ganglion in each of the segments XIII through XVIII, are connected to a dorso-lateral pair of vasa deferentia. Each vas deferens proceeds anteriorly to the region anterior to ganglion XIII where it enlarges and becomes the severely convoluted, sperm-filled epididymis. Narrowing again, the vas deferens extends anteriorly to ganglion X where the lumen greatly enlarges into the sperm-filled ejaculatory bulb (spermiducal gland of Hoffman, 1964), which is also severely convoluted (Fig. 3, A). The convolutions of the ejaculatory bulb extend as far anteriorly as the posterior part of segment IX. The lumen becomes confluent with the ejaculatory duct which proceeds posteriorly as well as ventro-medially where it enters the ventro-medial part of the rounded atrial cornu (Fig. 3, E) at ganglion XI. The lumina of the paired atrial cornua proceed ventro-medially to become confluent in the large medial atrium (Fig. 3, C). The lumen of the atrium

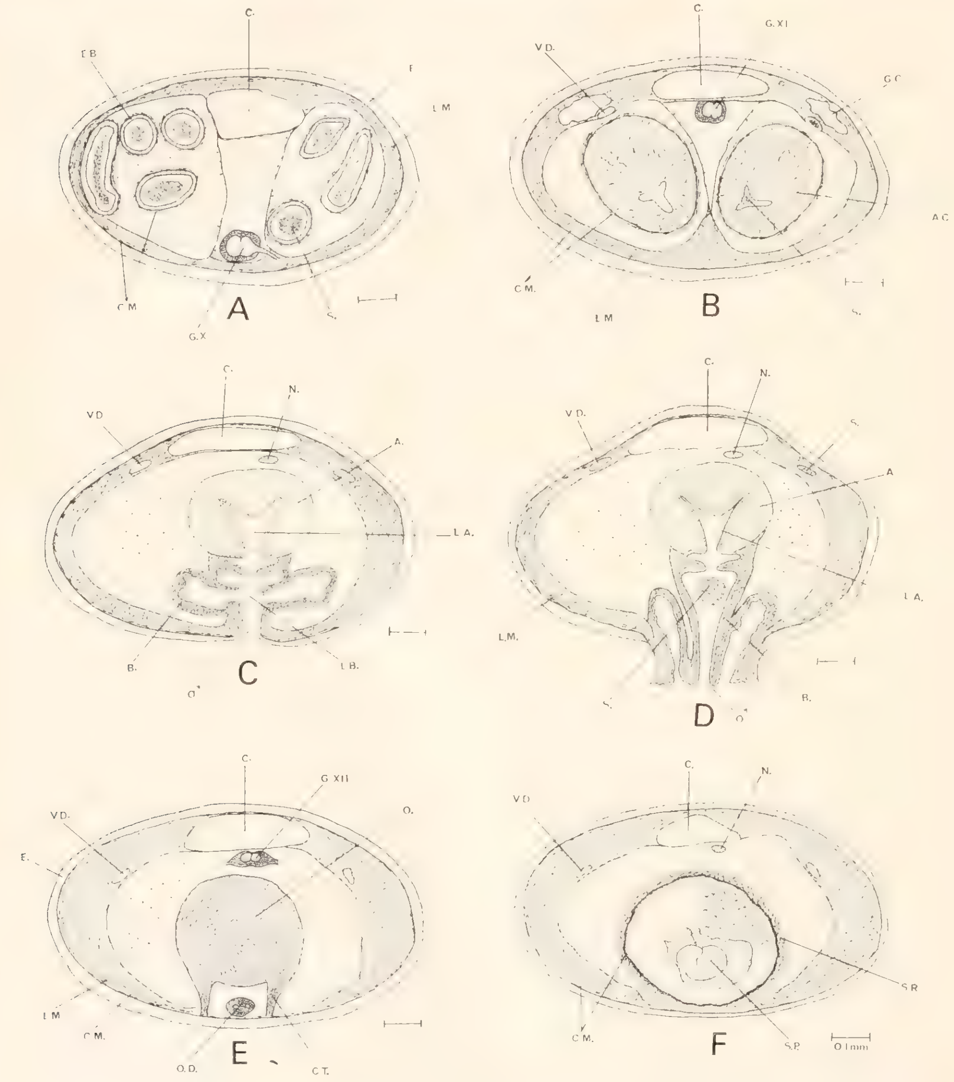

FIGURE 3. *Calliobdella carolinensis;* cross-sections through various regions of the reproductive system; A, ejaculatory bulb, at region of ganglion X; B, atrial cornua, at region of ganglion XI; C, bursa (not everted), immediately posterior to ganglion XI; D, bursa everted to form an intromittent structure (based on a specimen fixed *in coitu*), immediately posterior to ganglion XI; E. ovisacs, at region of ganglion XII; F, seminal receptacle, anterior to ganglion XII; a, atrium; a.c, atrial cornu; b, bursa; c, crop; c.m, circular muscles; c.t, conducting tissue; e, epidermal cells; e.b, ejaculatory bulb; g, ganglion; g.c, gut cecum; l.a, lumen of atrium, l.b, lumen of bursa; l.m, longitudinal muscles; n, nerve cord; o, ovisacs; o.d, oviduct; s, spermatozoa; s.p, spermatophore; s.r, seminal receptacle; v.d, vas deferens.

bends ventrally to become confluent at XI/XII with the lumen of the spacious, saccular bursa (Fig. 3, C), which in turn leads to the male gonopore. The non-glandular bursa is capable of being protruded externally to form a true copulatory organ (Fig. 3, D). We have observed true copulation in this species on numerous occasions.

The anatomy of the reproductive system in *C. carolinensis* is remarkable in having, from XI/XII to immediately posterior to ganglion XII, an unpaired "seminal receptacle" (Fig. 3, F), which probably corresponds with the paired vesiculae seminales of Johansson (1896) and Leigh-Sharpe (1914). The anterior portion of the lumen of this structure is lined by a thin layer of secretory cells which are completely encircled by a layer of circular muscles. The extreme posterior portion of the slightly bilobed seminal receptacle is continuous with the cells (possibly vector tissue) of the anterior portion of the female reproductive system (Fig. 2, s. r.). Sections from a specimen fixed *in coitu* revealed a true seminal receptacle containing numerous spermatozoa and a structure which appears to be a spermatophore (Fig. 3, F, s. p.) : a bilobed aggregation of sperm cells, surrounded by a non-cellular, strongly eosinophilic area. Unlike the similar "spermatheca" found in *Marsipobdella sacculata* Moore, 1952, there is a broad connection between the lumen of the seminal receptacle and the male bursa.

The female gonopore is inconspicuous in cross-section and difficult to discern externally. The paired ovisacs (Fig. 3, F), which extend from ganglion XII to ganglion XIII, inclusive, contain many immature ova. Although the mid-ventral epidermis in the region of the female gonopore is remarkably thin in the individuals sectioned, no distinct copulatory zone could be distinguished externally. In some of the cross-sections a pair of conducting strands occur on either side of the oviduct from near the female gonopore to the posterior portion of the seminal receptacle. In addition, the cells from the posterior part of the seminal receptacle are continuous with the anterior part of each of the ovisacs. The exact relationship between the seminal receptacle and both the oviduct and ovisacs has not been further determined, but the structures are remarkably similar to those of *Calliobdella lophii* Van Beneden and Hesse, 1863, and *C. nudulifera* (Malm, 1863) figured by Johansson (1896, Fig. 15), Brumpt (1900, Fig. 8), and Leigh-Sharp (1914, Fig. 3).

A definite basophilic epithelium surrounds the lumen of the vas deferens, the bulbus ejaculatorius, the ductus ejaculatorius, the medial atrium, the bursa and portions of the seminal receptacle. The secretory cells of the atrial cornua and the medial atrium are columnar and have strongly eosinophilic granules, whereas those of the bursa and the seminal receptacle are more irregularly shaped and have basophilic granules. No columnar, granular cells occur in the vas deferens, the ejaculatory bulb or the ejaculatory duct. In the immature specimen (3.0 mm) all parts of the reproductive system are poorly developed and strongly basophilic. A solid aggregation of cells, which may correspond to the seminal receptacle of the adult, extends posteriorly from the bursa to the solid, bilobed ovaries, which terminate at ganglion XII.

In adult *C. carolinensis* large clitellar gland cells with deeply eosinophilic secretory granules occur between the gut and the body wall, primarily in segments XIII through XXII. In the immature specimen the granules of the clitellar glands do not stain with eosin. The secretory ducts from these glands can be traced from ganglion XVII to the clitellar segments (X through XII) where the ducts (6–10 mu in diameter) project through the body wall. From ganglia XIV through XV these secretory ducts aggregate into three pairs of bundles: a small dorsal, a large dorso-lateral, and a large ventro-lateral bundle. In segment XIII the last bundle divides into two: a ventral pair and a ventro-lateral pair. Secretory cells resembling these clitellar gland cells (probably the pre-clitellar gland cells of other piscicolids) and their ducts occur in segments VIII and IX.

ECOLOGY: *C. carolinensis* is known to occur in South Carolina estuaries from Beaufort to Georgetown. Around Charleston *C. carolinensis* has been found in salinities from 4‰ to 32‰ during the months of January through April. Although often found free-living on *Ulva lactuca* and other chlorophytes, it feeds primarily on two species of clupeid fish, the Atlantic menhaden, *Brevoortia tyrannus,* and the blueback herring, *Alosa aestivalis.* A more detailed account of the biology of the species will be published elsewhere.

DISCUSSION

Based on superficial characters alone, *Calliobdella carolinensis* definitely belongs to the freshwater *Piscicola-Cystobranchus,* and the marine *Calliobdella-Trachelobdella* complexes of genera, all of which (except some *Trachelobdella*) possess six pairs of testisacs, two pairs of eyes on the oral sucker, external pulsatile vesicles, fused posterior crop ceca and an elongated, subcylindrical body, narrower than the caudal sucker. The structure of the male and the female reproductive systems closely resembles the European marine *Calliobdella,* which was reviewed by such early workers as Johansson (1898) and Stshegolew (1912). The unique reproductive structure in this genus was examined in depth by Johansson (1896), Selensky (1915) and Brumpt (1900). In *Calliobdella carolinensis* the weakly muscularized "seminal receptacle" which leads posteriorly from the bursa and is confluent with the conductive tissue of the female system corresponds with a similar but less well developed structure found in both species of *Calliobdella, C. lophii* and *C. nodulifera.* Selensky (1915) in referring to this structure writes: "Strands of the conducting tissue come to the posterior wall of the bursa, in the place of their contact with the epithelium arises the copulation area. We will note besides that the pair of longitudinal strands anastomize in two places and discharge into the bursa 'tubes' which were described formerly by Johansson (1896, 1898) as 'vesiculae seminales' are evidently nothing but the mentioned traces of the conducting tissue." The sperm-filled "seminal receptacle" of *C. carolinensis* is slightly paired at its extreme posterior end, which is evidently an elaboration of the paired "vesiculae seminales" noted by Johansson. In addition to differing from its congenitors in the shape and size of the seminal receptacle, *C. carolinensis* differs in the proportions of the body, the oral and caudal suckers, the host upon which they feed and the ecological conditions in which they occur.

We thank Donald L. Hammond and Charles H. Farmer, both of the South Carolina Marine Resources Division, for their invaluable assistance in collecting alive so many specimens of the leech described above.

SUMMARY

1. A new species of marine leech is reported from the estuaries of South Carolina on the clupeid fishes, the Atlantic menhaden, *Brevoortia tyrannus* (Latrobe) and the blueback herring, *Alosa aestivalis* (Mitchill).

2. The leech, which is provisionally assigned to the genus *Calliobdella,* has the following characteristics: 14-annulate, two pairs of cephalic ocelli, no ocelli on caudal sucker, 11 pairs of pulsatile vesicles and a well-developed medial, muscular organ which functions as a seminal receptacle.

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EVIDENCE FOR A SPONTANEOUS OVARIAN CYCLE IN FISH OF THE GENUS *XIPHOPHORUS*[1]

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The reproductive cycle of fish of the genus *Xiphophorus*, as well as certain other poeciliid fishes, has been described by Bailey (1933) and Turner (1937). These viviparous Teleosts were found capable of producing broods throughout the year. After insemination by a male, sperm usually continue to survive in the folds of the female ovarian tissue and as many as 8 successive broods can be produced by one insemination (VanOordt, 1928). Vallowe (1953) calculated a mean interval of 33.5 days between broods of *Xiphophorus maculatus* (platyfish). The calculation of the number of days from insemination to the dropping of the first or primary brood depended on his ability to observe successful copulations. He determined the mean number of days to the dropping of 19 primary broods to be 41.7 and suggested that some factor, associated with the ovary, was involved in delaying the birth of primary broods. Similar results have been reported for the guppy *Poecilia reticulatus*, by Rosenthal (1952) who attributed the wide variation in time for the production of primary broods to an "ill-defined" estrus cycle.

The nature of this cycle should be distinguishable by utilizing artificial insemination to precisely fix the moment of insemination and to produce large numbers of primary broods. By plotting the length of time required to produce primary broods against the time to produce subsequent broods (as suggested by J. W. Atz, American Museum of Natural History, personal communication), the nature of the ovarian cycle operating in these fish could be more adequately analyzed. As part of a study in which it was necessary to produce large numbers of hybrids between fish of the genus *Xiphophorus*, such a procedure was carried out and is reported here.

MATERIALS AND METHODS

All fish were the descendants of fish obtained from the Fish Genetics Laboratory of the New York Zoological Society in 1963. F_1, F_2 and backcross hybrids were produced between descendants of the swordtail, *Xiphophorus helleri strigatus* (Regan) originally collected from the Rio Sarabia, and the platyfish, *X. maculatus* (Guenther) strain 163A originally collected from the Rio Jamapa. Most matings were performed using the artificial insemination technique described by Clark

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(1950). Some F_2 and backcross hybrids were produced naturally. Inseminations were performed randomly with respect to cross type and season of the year, over a 3-year period. Female fish artificially inseminated were in their second year of life and had been kept isolated from males since maturity.

Each artificially inseminated female fish was kept in a 5-gallon aquarium filled with conditioned tap water which contained an abundance of the filamentous alga,

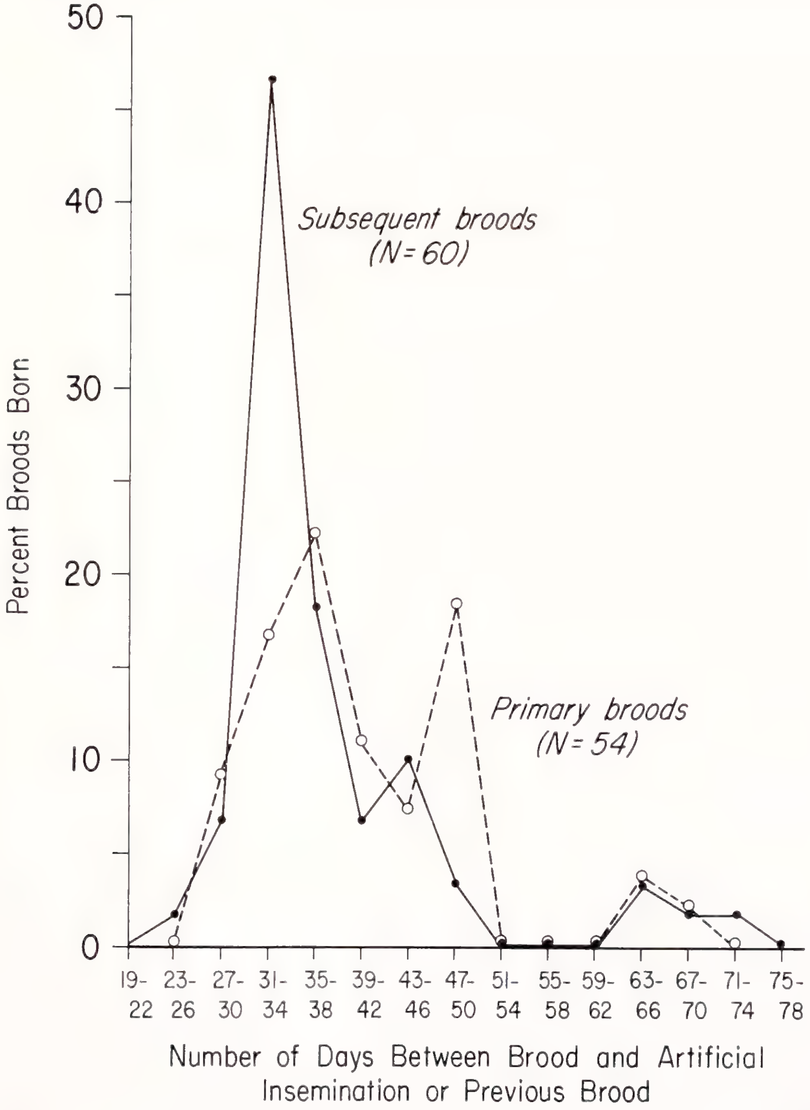

FIGURE 1. Percentage of primary broods born in 4-day intervals from 19 to 78 days following artificial insemination and subsequent broods born during the same intervals following the birth of previous broods.

Nitella. The water was under constant aeration and its temperature was maintained at 24° C ± 3°. No attempt was made to artificially control light. After birth of a brood (primary brood), the parent was immediately removed to another tank to await the birth of subsequent broods. The date each brood was dropped was recorded and the number of days between insemination and each primary brood was calculated. The date of each subsequent brood was recorded and the number of days between each brood was also calculated and compared to the number of days between insemination and each primary brood. Subsequent broods resulting from some natural matings were included in the data used to calculate the number of days between broods.

The percentage of primary broods dropped in 4-day intervals from 23 to 74 days after artificial insemination was scored and compared with the percentage of subsequent broods dropped during the same intervals following the birth of a previous brood. Any differences in the percentage of primary *versus* subsequent broods during such a particular time period were tested for statistical significance by making use of the binomial probability distribution (Sokal and Rohlf, 1969, pages 65–98). All such calculations were performed on a high-speed computer.

The mean number of days to the dropping of broods in various categories was compared and analyzed for statistical significance by the Student's t test.

RESULTS

A total of 54 primary and 60 subsequent F_1, F_2, backcross to the swordtail (XXP-BC), and backcross to the platy (XPP-BC) broods were produced. The number of days between artificial inseminations and the dropping of primary broods, and the number of days between subsequent broods and previous broods, under the conditions of this laboratory, are compared in Figure 1. In addition to these broods, single primary broods were also dropped on the 79th, 91st, 106th, 155th, and 181st days following artificial inseminations.

The highest number of subsequent broods was dropped from 31 to 34 days after a previous brood (subsequent peak period). A peak number of primary broods was dropped from 35 to 38 days after artificial insemination (first primary peak) and again from 47 to 50 days after artificial insemination (second primary peak). The percentage of primary broods born during the second primary peak time period is significantly higher than the percentage of subsequent broods born during the equivalent time period ($P < 0.01$). The same is true for the percentage of subsequent broods dropped during the subsequent peak period with respect to the percentage of primary broods born during the equivalent time period. In no other time categories does the percentage of primary *versus* subsequent broods differ significantly.

Almost all broods were dropped from 23 to 50 days following artificial insemination or the birth of a previous brood. The mean number of days for all broods born within this time period was 36.6 ± 0.6. The mean for primary broods was 38.3 ± 1.0 days while the mean for subsequent broods was 35.1 ± 0.7 days. The difference between these means is highly significant ($P < 0.01$).

Primary broods dropped during the later portion of the 23- to 50-day time period (37–50 days after artificial insemination) are listed, along with other data relative to the inseminations, in Table I. Similar information is contained in

Table II for primary broods dropped during the early portion of the 23 to 50 day time period (23 to 36 days after artificial insemination).

The mean number of days to the dropping of subsequent broods from females who dropped their primary broods during the later portion (37–50 days after artificial insemination) of the 23- to 50-day time period (34.8 ± 1.2) was not statistically significant when compared to females who dropped their primary broods during the early portion (23 to 36 days after artificial insemination) of the 23- to 50-day time period (35.4 ± 0.8).

TABLE I

Primary broods born from 37 to 50 days following artificial insemination

Insemination number	# Days to birth of primary brood (x_1)	# Days to birth of subsequent broods (x_2)	Female parent	Type of hybrids produced	Month/day of insemination
22	45	—	Platy	F_1	2/28
30	49	31, 31	Swordtail	XXP-BC	6/7
31	49	—	Swordtail	XXP-BC	6/7
32	49	—	Swordtail	XXP-BC	6/7
33	49	32, 31	Swordtail	XXP-BC	6/7
34	47	35, 48, 40	F_1	F_2	2/13
36	47	31, 30	F_1	F_2	2/13
41	47	—	Swordtail	XXP-BC	2/1
43	49	—	Swordtail	XXP-BC	6/24
58	40	—	Platy	F_1	11/17
61	41	33, 32	F_1	XPP-BC	11/17
64	37	—	F_1	XPP-BC	4/11
66	43	37, 41	Swordtail	XXP-BC	11/17
67	45	—	Swordtail	XXP-BC	11/18
68	40	33	Platy	XPP-BC	11/18
82	49	35, 46	F_1	XXP-BC	11/19
86	50	31	F_1	F_2	11/25
95	38	33	Platy	F_1	4/4
97	41	—	Platy	F_1	4/10
113	40	30, 35	F_1	XPP-BC	8/20
114	40	—	F_1	XPP-BC	8/20
117	43	—	F_1	XPP-BC	8/20
—	—				
Means	$\bar{x}_1 = 44.5 \pm 0.9$	$\bar{x}_2 = 34.8 \pm 1.2$			
	$t = 6.6, P < 0.01$				

DISCUSSION

The data are consistent with the observations made by Vallowe (1953) that the mean time to the first or primary brood is longer than the mean interval between subsequent broods in fish of the genus *Xiphophorus*. However, by using artificial insemination to precisely fix the moment of sperm introduction in a large number of broods and by plotting the data modally, a general lag to the production of primary broods is not observed. Primary broods were dropped in two peak periods (1st and 2nd primary peaks). The time of the 1st primary peak roughly corresponds to the peak period in which subsequent broods were dropped. Therefore, the major reason for the higher mean time for the dropping of primary broods, must have been due to the broods dropped in and around the second primary peak. The fact that the percentage of primary broods dropped within this period was significantly higher than the percentage of subsequent broods dropped from 47–50 days after the birth of a previous brood indicates that the second primary peak is present for reasons other than random variation of the gestation time of indivdual broods in the two groups.

It could be argued that there may be a nonrandom difference in the gestation time of the fish that dropped primary broods in and around the second primary peak as opposed to the fish which dropped primary as well as subsequent broods earlier. This could be considered a manifestation of the time of year these inseminations were carried out or the type of hybrids produced in the brood. However, that this is not the case is indicated by the data in Tables I and II. Primary broods dropped during the later portion of the 23- to 50-day period (37–50 days after artificial insemination) present the same spectrum of F_1, F_2, XXP-BC, and XPP-BC hybrids, and were inseminated at equivalent times of the year as those dropped during the earlier portion. That these fish do not have abnormally long gestation periods is indicated by the fact that the length of time to the dropping of their subsequent broods is equivalent to the interval

TABLE II

Primary broods born from 28 to 36 days following artificial insemination

Insemination number	# Days to birth of primary brood (x_1)	# Days to birth of subsequent broods (x_2)	Female parent	Type of hybrids produced	Month/day of insemination
1	30	—	Swordtail	XXP-BC	5/6
4	28	—	Platy	F_1	10/20
6	31	—	Platy	F_1	10/20
8	34	36	Swordtail	F_1	10/27
12	35	34	Platy	F_1	1/13
13	32	37	Swordtail	F_1	1/13
25	35	—	Swordtail	F_1	3/28
29	35	30	Swordtail	F_1	5/16
39	35	—	F_1	XPP-BC	2/1
40	35	33, 35, 35, 41	F_1	XPP-BC	2/1
45	28	34	Swordtail	XXP-BC	6/24
56	35	45	Swordtail	F_1	6/25
73	36	—	Swordtail	XXP-BC	11/18
75	31	33, 43, 31	F_1	XXP-BC	11/19
76	31	—	F_1	XXP-BC	11/19
77	36	—	F_1	XXP-BC	11/19
78	31	33, 43, 31	F_1	XXP-BC	11/19
80	31	38, 32, 37	F_1	XXP-BC	11/19
104	35	—	Platy	XPP-BC	6/19
116	33	—	F_1	XPP-BC	8/20
121	30	32, 35	Swordtail	F_1	8/20
123	35	33, 37	Swordtail	F_1	8/20
128	28	—	Platy	F_1	10/15
129	34	32	Platy	F_1	8/29
Means	$\bar{x}_1 = 32.7 \pm 0.6$	$\bar{x}_2 = 35.4 \pm 0.8$			
	$t = 8.7$, $P < 0.01$				

between broods of fish whose primary broods were dropped during the earlier portion of the 23- to 50-day period. Primary broods dropped in and around the second primary peak must have been the results of fertilizations which were delayed independently of time of year and cross type. Therefore, in some inseminations (indeed most) fertilization was quick and in others, delayed.

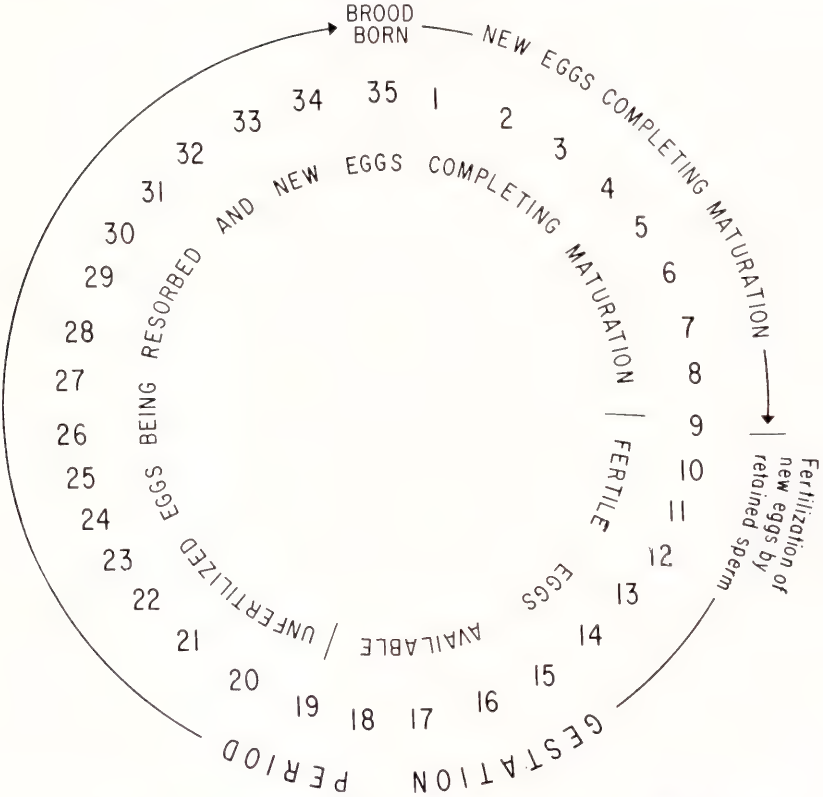
BROOD BORN
NEW EGGS COMPLETING MATURATION
Fertilization of new eggs by retained sperm
GESTATION PERIOD
NEW EGGS COMPLETING MATURATION
FERTILE EGGS AVAILABLE
UNFERTILIZED EGGS BEING RESORBED AND
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FIGURE 2. Model spontaneous ovarian cycle for fish of the genus *Xiphophorus*. The days for a typical cycle in the fish used in this laboratory are indicated by the numbers. The events which take place in an impregnated fish are indicated peripheral and adjacent to the days of the cycle in which they occur. The events which take place in a virgin fish are indicated inside and adjacent to the appropriate days of the cycle in which they occur.

The presence of a single peak period (conforming to the mean number of days after dropping of previous brood) for the dropping of subsequent broods is in conformity with brood interval data reported by numerous investigators. The mean number of days to the dropping of subsequent broods reported here (35.1 ± 0.7) is higher than that reported in the past and is most likely a reflection of the relatively constant cool temperatures maintained.

The finding that primary broods are born in two peak periods, an earlier one conforming to the subsequent peak period and a later one from 47–50 days after artificial insemination, is not inconsistent with the results of Vallowe (1953), Rosenthal (1952) or Clark (1950). Measuring the time from observed copulation, cohabitation with males, and artificial insemination, respectively, each of these investigators reported females dropping primary broods from a time approximating the interval between broods up to about 50 days after the initiation of the experiment. The relatively few number of reported cases and the relatively imprecise method of determining the moment of insemination (in Rosenthal's experiments) does not allow an analysis of peaks. It was suggested (Rosenthal, 1952) that the wide spread in time for the production of primary broods was the result of an ill-defined estrus cycle—ova mature and in the absence of fertilization are periodically resorbed.

An interesting result is seen in Clark's data. Although she only reported 9 successful artificial inseminations, in one of them a brood was produced in only 22 days. The earliest subsequent brood obtained was dropped by the same fish in 26 days, followed by another in 27 days. In the present experiment, conducted under highly controlled temperature conditions, the earliest brood was a subsequent, not a primary. However, as indicated in Table II, the mean time to the dropping of primary broods born in less than 37 days after artificial insemination was significantly less than the mean interval between broods in those fish. Ten of 13 of these fish which dropped subsequent broods produced their primary broods in less time than at least one of their subsequent broods. These results are interpreted as indicating that in many virgin fish, fertilization and the beginning of the gestation take place sooner after artificial insemination than they do after the birth of a previous brood.

These observations can be explained by the presence of a spontaneous ovarian cycle which is present in all mature female fish—virgin or inseminated. The length of this cycle, which is temperature dependent, can be measured by the interval between broods. Under the conditions of this laboratory the typical cycle was 35 days (Fig. 2). One may consider a female who has dropped a brood, to have dropped it on the 35th day of the cycle. The next day would mark day "1" of the next cycle. Since Tavolga (1949) calculated the completion of egg maturation to take 6.8 days following the birth of a brood when the brood interval was 28 days, in the present study the egg maturation period is considered to be 8 days followed by a 27-day gestation period. A fish may therefore be considered to contain mostly fertile eggs starting on the 8th day of the cycle. (Turner 1937, pointed out that all eggs, and consequently all embryos, are not at the same maturation stage at the same time. However, as Tavolga noted in 1949, they all are similarly developed at birth because of the slowness of the last embryological steps.) Eggs capable of being fertilized would be present in the ovary for the next 11 days through day 19 of the cycle (explained below). If not fertilized, the eggs would be resorbed followed by replacement with new eggs. These processes would consume 24 days lasting through the remaining 16 days of the cycle and into the first 8 days of the next cycle. It is suggested that this 35-day cycle is repeated during the mature period of a female fish whether or not it is ever fertilized.

Such a model spontaneous ovarian cycle explains the production of two peak periods of brood birth following artificial insemination of virgin fish. Primary broods born during the first half of the 23- to 50-day period following artificial insemination would theoretically conform to inseminations taking place during the first 19 days of the female's cycle. Many of these broods were dropped in less time than subsequent broods from the same female. Such a situation would be accounted for by inseminations taking place from day 8 of the cycle to day 19 (assuming most eggs were mature and available for fertilization).

Primary broods born during the latter half of the 23- to 50-day period would conform to inseminations taking place from the 20th to 35th day of the cycle. Sperm introduced at this point in the cycle would have no fertile eggs with which to combine. This sperm would be stored and be available to fertilize eggs, some of which would be ready on the 8th day of the next cycle. Such broods would be born at a minimum of 36 days after insemination (inseminations on the 34th day of the cycle) to a maximum of 50 days after insemination (inseminations on the 20th day of the cycle). Primary broods should not take longer than 50 days after artificial insemination since sperm introduced before the 20th day of the cycle should be in time to fertlize good eggs produced earlier in that cycle. The fact that 3 primary and 4 subsequent broods were seen from 63 to 74 days after artificial insemination or birth of a previous brood may be interpreted as broods which were missed or skipped a cycle. Primary broods dropped on the 79th, 91st, 106th, 155th and 181st days following artificial insemination were very likely from late maturing females.

A basic feature of this model is that fertile eggs were available for fertilization for only approximately 11 days (day 9 through 19 of the cycle). Why 11 days? If less than 11 days, 6 for instance, then fertile eggs would not be available for fertilization of the 16th, 17th, 18th and 19th days of the cycle. Sperm introduced at those points would have to wait 28, 27, 26 and 25 days, respectively, for fertile eggs to become available in the next cycle. These delays plus the 27 day gestation period would mean that broods in those cases would not be dropped until the 55th, 54th, 53rd and 52nd days following artificial insemination. Since no primary broods were born from 51 to 62 days following artificial insemination (Fig. 1), eggs capable of being fertilized must have been present through the 19th day of the cycle and therefore at least 11 days. If fertile eggs were present for more than 11 days, 16 for instance, they would have been available for fertilization on the 20th, 21st, 22nd, 23rd and 24th days of the cycle. This would mean that the earliest day of the cycle in which fertile eggs were not available would have been day 25. Sperm introduced on that day would have to wait only 19 days for fertile eggs to become available. Considering the 27 day gestation period, the maximum time for primary broods to be dropped following artificial insemination would have been 46 days. However, fully 18.5% of the primary broods were born from 47–50 days following artificial insemination (Fig. 1). Therefore, these broods appear to be the result of inseminations from the 20th to 24th days of the cycle during which time fertile eggs were not available.

An alternate interpretation could also be applied to these data. Perhaps a female fish which had not recently reproduced, possessed eggs which were not capable of being fertilized. Inseminations would then stimulate the resorption of these eggs and production of a new set. Such activity would account for a second primary peak independent on a spontaneous cycle. The first primary peak would be the result of inseminations into fish in which the eggs had not yet become overripe.

While further work is necessary to resolve these two interpretations, it is felt that the following observations speak in favor of the spontaneous cycle in fish of this genus: (1) Tavolga (1949) has noted that unfertilized degenerating eggs are of frequent occurrence, particularly in *virgin* females. (2) As pointed out by Turner (1937) several species in this family display superfoetation (more than one brood of embryos of different sizes in the ovary at the same time). Such a phenomenon is indicative of the fact that a spontaneous ovarian cycle is not strange to the family *Poeciliidae*.

SUMMARY

1. The nature of the ovarian cycle in fish of the genus *Xiphophorus* was studied by using artificial insemination to precisely fix the moment of sperm introduction and by comparing the time to produce primary broods with subsequent broods.

2. The mean time to the production of primary broods was significantly longer than the time to produce subsequent broods. However, there was not a general lag in the production of primary broods but instead they were seen to be born in two peak periods—an earlier one conforming to the single subsequent brood peak, and a later one.

3. The presence of two primary peaks, the fact that the mean number of days to the production of those broods clustered around the first peak was significantly less than that of their subsequent broods, and the lack of the birth of any broods in the time periods immediately following the second peak suggests the presence of a spontaneous ovarian cycle in these fish.

4. Based on the time factors observed in this laboratory, a model cycle was constructed indicating its length (35 days), gestation period (27 days), and length of time fertile eggs were available in the ovary (11 days).

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STUDIES ON THE DEVELOPMENT OF THE SEA URCHIN *STRONGYLOCENTROTUS DROEBACHIENSIS*. III. EMBRYONIC SYNTHESIS OF CILIARY PROTEINS

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The formation of cilia at the blastula stage represents an important morphological event in the development of echinoderm and other embryos. Parthenogenetic activation leads to normal (but haploid) division, blastula formation, subsequent ciliogenesis, and indeed to full larval development and metamorphosis, indicating that maternal genetic information and organelles are quite sufficient for full development, and that the male nucleus, centriole, and basal body are superfluous (*cf.* Wilson, 1925). Parthenogenetic activation of enucleate half-eggs of the sea urchin leads to cytokinesis, blastula formation, and hatching, but to no further development, indicating that maternal cytoplasmic information is sufficient for at least the mechanics of pre-gastrular development (Harvey, 1940). The formation of cilia during enucleate parthenogenetic development has been reported (Harvey, 1940), but this highly important claim has not yet been substantiated by electron microscopy. Early development through the point of cilia formation and hatching takes place in the presence of sufficient actinomycin D to totally abolish RNA synthesis, indicating a stable masked messenger RNA template (Gross, Malkin, and Moyer, 1964; Guidice, Mutolo, and Donatuti, 1968). After "deciliation", normal regeneration of cilia can occur in the presence of Actinomycin D (Auclair and Siegel, 1966), without DNA-dependent RNA synthesis. These same workers also demonstrated that multiple cilia regenerations could take place in the presence of puromycin although the synthesis of ciliary proteins was reduced by 90%, thus indicating an extensive pool of ciliary precursors at least at the gastrula stage. The repeated formation of cilia in the near absence of transcription and translation would point to a self-assembly process from pre-existing active precursors. Pulse-labeling of developing sea urchin embryos and the subsequent isolation of cilia indicate a fairly steady rate of total ciliary protein synthesis prior to and early in ciliogenesis (Auclair and Meismer, 1965; Stephens, unpublished). If cilia formation is a self-assembly process and if the ciliary proteins are made prior to ciliogenesis, what then triggers this precisely-timed morphological event? Is some critical component synthesized immediately prior to organelle assembly in such small amount that it is overshadowed by the bulk proteins of the cilium, or is there some initiating enzymatic activation process whereby modification of the pre-existing components brings about a "crystallization" of all of the requisite proteins into a cilium?

This present report attempts to answer some of these questions through a fractionation of ciliary axonemes, obtained from pulse-labeled sea urchin embryos, into known structural components, followed by an analysis of the sequence of synthesis of such components.

Materials and Methods

Embryonic material

The sea urchin *Strongylocentrotus droebachiensis* was obtained from the Supply Department of the Marine Biological Laboratory. Gametes were shed, egg jelly coats were removed, and the eggs were fertilized and grown at 7.5° ± 0.2° C according to the methods outlined in full elsewhere (Stephens, 1972a). All operations were conducted at or below the growth temperature to avoid temperature shock and assure synchrony. Sterile sea water containing 0.05% sulfadiazine was used to initially wash the eggs and was used in all subsequent egg suspensions.

Experimental design

In a typical experiment, 12 ml of dejellied eggs from one female were fertilized in 250 ml of sea water at zero time. An aliquot of egg suspension containing 1 ml of eggs was immediately withdrawn, spun down in a hand centrifuge, and 5.0 ml of sea water containing 4 μc/ml of ^{14}C-leucine were added. This incubation mixture was shaken gently during a three hour pulse period. At the end of this period, the eggs were spun out of the pulse solution, resuspended in 10 ml of sea water chase containing 1% cold leucine, and agitated for an additional 30 minutes. The embryos were again gently spun down, resuspended in 100 ml of normal sea water, and allowed to develop undisturbed through cilia formation and hatching. Additional aliquots were taken at successive 3 hour intervals through 30 hours of growth and all treated in an identical manner. The net result of this protocol was to produce 11 aliquots of ciliated blastulae, differing only in the time-point of pulse labeling. With adequate temperature control, all embryos hatch within a 15–30 minute interval.

Ciliary axoneme preparation

Cilia were isolated 12 hours after hatching by transferring the blastulae to 10 ml of ice-cold hypertonic sea water (containing an additional 30 g/l NaCl, Auclair and Siegel, 1966) and stirring gently for 2 minutes. The deciliated embryos were immediately centrifuged and resuspended in fresh normal sea water for regeneration studies. The supernatant from the deciliation was centrifuged at 10,000 × g for 10 minutes to sediment the cilia. The resultant pellet was suspended in 5 ml of 1% Triton X-100 detergent containing 30 mM Tris HCl, pH 8.0, and 3 mM $MgCl_2$ (Stephens, 1970a) for 30 minutes to remove the ciliary membranes, matrix, and any accidental cell debris. The ciliary axonemes were then sedimented at 10,000 × g for 10 minutes and the supernatant was discarded. Phase-contrast microscopy of the pellet (Fig. 1) indicated only the presence of ciliary axonemes; no cell fragments or bacteria were observed. Regenerated cilia were isolated in an identical manner from the above resuspeneded deciliated embryos after an 8-hour regeneration period.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

To each ciliary axoneme pellet produced above, 0.2 ml of 1% sodium dodecyl sulfate (SDS) containing 10 mM phosphate, pH 7.0, 1% 2-mercaptoethanol, and 10% glycerol was added and then heated to 90° C for 2 minutes. Gel electrophoresis was carried out by the methods of Shapiro, Vinuela, and Maizel (1967) on 100 microliter duplicate aliquots of the dissolved axoneme sample (initial volume of about 0.3 ml) applied to 10 cm length 5% acrylamide gels and run at 50 volts. The gels were stained for 2 hours with at least 10 volumes of Fast Green (Gorovsky, Carlson, and Rosenbaum, 1970) at a concentration of $\frac{1}{2}$% in 50% methanol-10% acetic acid (Stephens, 1972b), and then diffusion-destained by multiple changes of 7% acetic acid. Parallel gels of known molecular weight proteins (bovine serum albumin, tubulin, actin, and dynein) were run to calibrate the system. Bovine serum albumin applied to parallel gels in 5, 25, 50, and 75 microgram amounts stained in accord with Beer's Law over this range; ciliary axonemes were always applied at a concentration less than 50 micrograms. The analytical gels were then either photographed or else subjected to direct microdensitometry with a Joyce-Loebl MK III double beam recording densitometer. The amount of protein applied to each gel, as judged either from direct protein determination or from integrated microdensitometer tracings of stained gels, was identical within the limits of reproducibility of these methods (±15%).

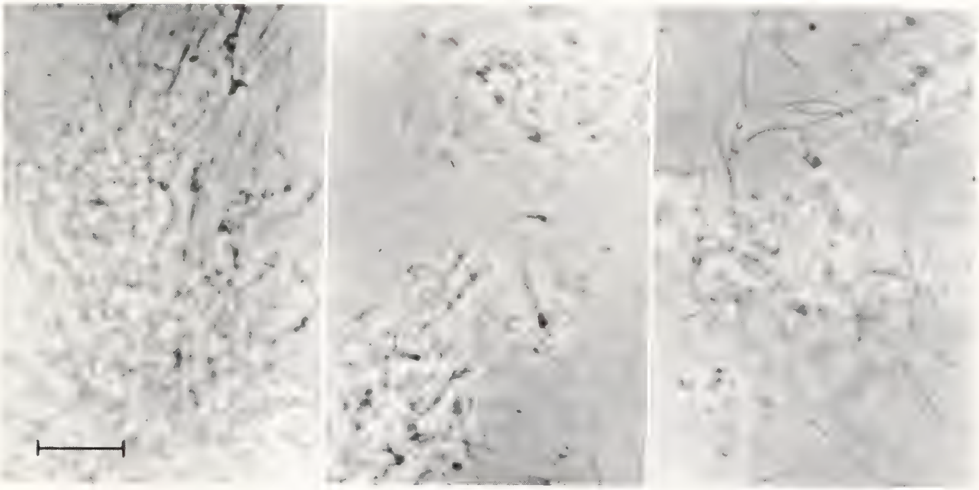

FIGURE 1. Phase-contrast micrographs of Triton X-100 extracted ciliary axonemes under oil immersion Leitz optics. Removal of the ciliary membrane causes aggregation of the axonemes. Scale marker equals 10 microns.

Protein concentration

Total embryonic, ciliary, or axonemal protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard. Determination from SDS-solutions was carried out by initially dissolving the sample in the *absence* of 2-mercaptoethanol, removing an aliquot of 25 microliters for protein determination, and then adding the mercaptoethanol from a micropipette prior to heating the sample. After electrophoresis, relative proportions of proteins in mixtures were estimated from the microdensitometer trace of a stained gel, assuming equal color yield for all components.

Autoradiography

After microdensitometry, the gels were sliced longitudinally, dried *in vacuo,* and autoradiographed for 2 weeks on Kodak No-Screen X-ray film, exactly according to the procedures of Fairbanks, Levinthal, and Reeder (1965). By using the densitometric tracings of the unsliced gels of labeled and stained ciliary axonemes, the amount of protein and radioactivity applied to each gel, and the densitometric traces of the corresponding autoradiogram, the relative specific activities of the various major components could be easily calculated. Changes in activity were estimated by comparison of traces at different time points.

Scintillation counting

Radioactivity in the SDS-solutions of isolated axonemes and in the ^{14}C-leucine supernatant solutions before and after pulse-labeling were determined by counting 25 microliter aliquots in Beckman "Fluoralloy" liquid scintillation fluid, using a Beckman LS 250 counter. Appropriate corrections were made for efficiency.

Isotope

Uniformly-labeled ^{14}C-L-leucine (Schwarz-BioResearch) with a specific activity of 312 mc/mmole was diluted to a concentration of 4 μc/ml in sterile sea water and neutralized to pH 8.0. The final leucine concentration was 1.3×10^{-2} μmole/ml. For each pulse, 20 μc of total isotope was used per ml of eggs or roughly per 100 mg of total egg protein.

Data reproducibility

The entire experimental design, involving uptake data, pulse-chase labeling of first-generation cilia, regeneration of cilia from previously-labeled embryos, gel electrophoresis, and autoradiographic analysis, was carried out on two separate egg batches, one early and one late in the breeding season. The protocol yielded duplicate gels, each of which in turn produced duplicate gel slices for autoradiography; comparable time points yielded essentially superimposable densitometric tracings and both experiments yielded identical patterns of sequential synthesis.

Results

The overall pattern of embryonic and ciliary protein synthesis

Throughout embryogenesis beyond the first division, between 75% and 88% of the total added ^{14}C-leucine was incorporated into protein of the whole embryo, as determined by counting TCA-precipitated protein from either an aliquot of the post-chase embryos or of the ciliated embryos after hatching. No measurable difference could be demonstrated in these two cases, indicating the effectiveness of the chase (*cf.* Auclair and Siegel, 1966). Measurement of the label left in the pulse-label supernatant fluid yielded a value of 88–92% uptake for the three-hour pulse periods in all but the first division time point. During the first division, 80–83% of the label was taken up and about 75% of the amount of total embryonic protein was synthesized relative to the later divisions.

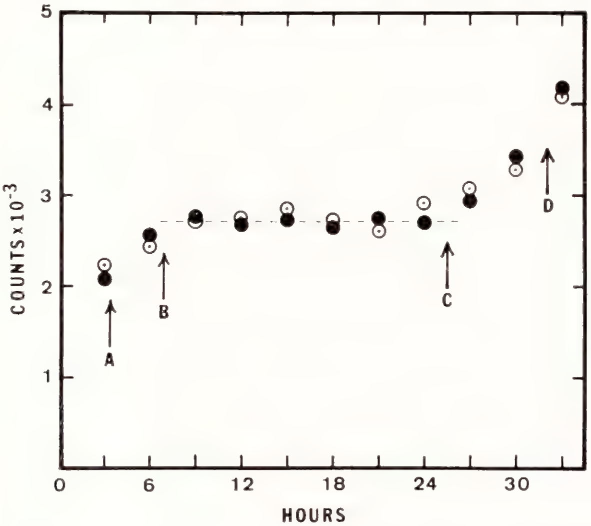

FIGURE 2. Incorporation of ^{14}C-leucine into ciliary axonemes after three-hour pulses. Various developmental stages are noted as follows: A—first division; B—8-cell stage; C—cilia formation begins; D—hatching. Specific activity is in counts per minute per microgram of axonemal protein. Experimental points are from two separate sets of experiments.

The isolated ciliary axonemes reflected this overall synthetic pattern, except during the process of ciliogenesis itself. Thus during the major portion of the early developmental process, regardless of the time point at which the pulse was introduced, the axonemes isolated after hatching had essentially the same specific activity. The only variation was an expected increase to this constant level after the first division and also a marked increase prior to hatching (Fig. 2). Thus the bulk of axonemal proteins are made before ciliogenesis as reported previously (Auclair and Meismer, 1965), but there is a marked increase in synthesis during the morphogenetic process. When whole cilia are counted after a similar pulse-labeling experiment, this increase is much less obvious; this is probably due to a masking effect since the cilia are at least twice as "hot" as the axonemes, contain twice as much protein, and this protein (chiefly membrane, matrix, and cell debris) is of nearly constant activity.

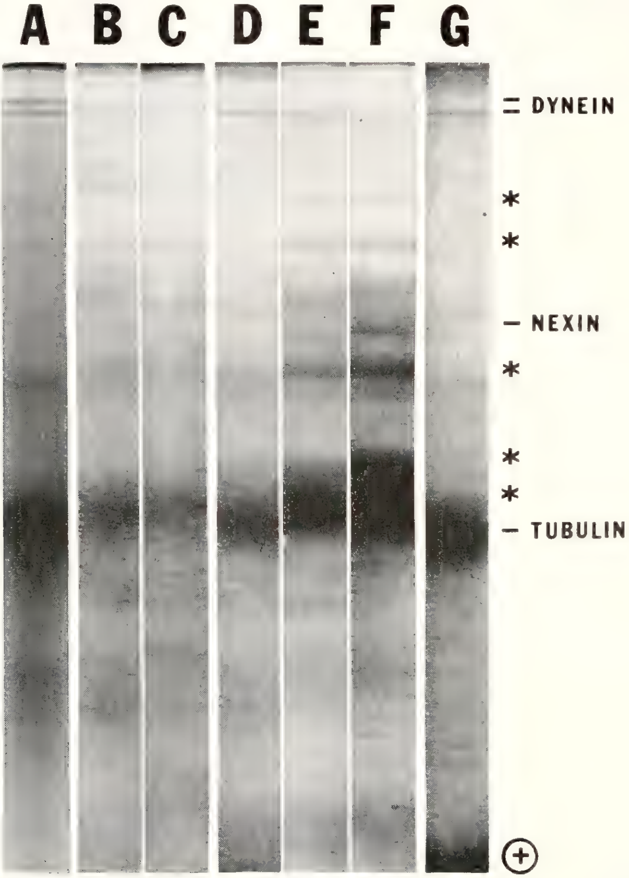

FIGURE 3. Acrylamide gel autoradiograms of ciliary axonemes. A—stained gel, 27–30 hour pulse period; B and C—autoradiograms of 6–9 hour and 12–15 hour pulse period gels; D, E, and F—autoradiograms of 24–27 hour, 27–30 hour, and 30–33 hour pulse period gels, respectively; G—autoradiogram of gel from regenerated axonemes from the 27–30 hour pulse period. Slight misalignment of bands is due to uneven drying of gels. Several bands are virtually indetectable in the stained gel, but are apparent in the autoradiogram due to high specific activity resulting from *de novo* synthesis (*cf.* Fairbanks *et al.*, 1965). All gels were loaded with the same amount of axonemal protein.

Over the moderately constant range of ciliary protein incorporation, the axoneme represents about 1.5% of the total incorporated radioactivity but accounted for only about 0.08% of the total embryonic protein by actual measurement after isolation. A very rough calculation of the expected amount of total axonemal protein, based on one 30 μ cilium per cell per 500-cell blastula in a sample of

125,000 blastula, gives a value of 0.1%. Thus during the pre-ciliogenesis period, the specific activity of the axonemal proteins average about 15–20 times higher than that of the embryonic proteins as a whole. Nearly ten-fold higher whole cilia values were reported by Auclair and Siegel (1966) for cilia regenerated from late gastrula.

Synthesis of specific proteins of the "9 + 2" structure

The data of Figure 2 would not indicate much in the way of a dramatic change in ciliary protein synthesis prior to or early in ciliogenesis, an event occuring at about 26 hours and thus within the 24–27 hour pulse. During active ciliogenesis, (the 27–30 hour pulse period) the specific activity increases by nearly 25% and during hatching, (the 30–33 hour pulse period) the specific activity increases to a value roughly 55% over the pre-ciliogenesis level. As noted above, and by previous workers, the bulk of the ciliary proteins are made prior to the actual formation of the organelle; the present data indicate a marked increase in incorporation during ciliogenesis. The question now becomes one of determining what these various proteins are and when they are synthesized. Analysis of SDS-electrophoretic autoradiograms of identical amounts of ciliary axonemes from embryos differing only in the time-point of the pulse-label shows that the proteins of the "9 + 2" axoneme fall into four general classes with regard to their timetable of synthesis.

Figure 3 includes photographs of autoradiograms for 6–9 hour and 12–15 hour pre-ciliogenesis pulse periods, 24–27 hours (representing initiation of cilia formation), 27–30 hours (active ciliogenesis), and 30–33 hours (representing final stages in ciliogenesis prior to and during hatching). Figure 4 is a composite diagram of autoradiographic microdensitometer tracings for 9–12 hour and 21–24 hour pre-ciliogenesis pulses and the 24–33 hour period of active ciliogenesis. These diagrams should be referred to in regard to the results discussed below.

(1.) *Pre-existing proteins*—The ciliary ATPase *dynein* (Gibbons, 1965 and 1966) is composed of two high molecular weight components in 2:1 ratio (Linck 1970 and 1971). Having revised molecular weights of 500,000 and 460,000 as determined through linear 3% acrylamide-SDS gel electrophoresis (Linck, 1971), these two prominent bands are easily observed on stained gels (Figs. 3A and 4, bottom trace). Upon autoradiographic analysis of such gels *no label is ever detected in the higher molecular weight dynein band*, regardless of pulse-label time. Weisenberg and Taylor (1968) report a 13S dynein-like ATPase protein in unfertilized urchin eggs, while Stephens (1972b) has detected only a *single* higher molecular weight band in the dynein region when whole eggs are subjected to SDS-acrylamide gel electrophoresis. The former authors speculated that the 13S ATPase may be a ciliary precursor; the protein synthetic data presented here indicates that it has to be since it is not synthesized after fertilization. Other minor components may also pre-exist but their amount would be too small to detect under the present methodology.

(2.) *Proteins synthesized at a constant pre-ciliogenesis rate*—As implied above, the *lower molecular weight component of dynein* is synthesized after fertilization; its rate of synthesis does not change even during the most active periods of ciliogenesis, as evidenced by its near-constant height and area in all of the autoradiograms (Fig. 4, all traces). Fractionation of ciliary axonemes into two equally active dynein fractions, one lacking the lower molecular weight component, would indicate that this component is structural rather than enzymatic (Linck, 1971 and in preparation). The *tubulin* fraction is also synthesized at a uniform rate beyond the first division; during active ciliogenesis, however, the estimated specific activity of total tubulin increases by about 50% (Table I). Fractionation of doublet tubules into A- and B-components (Stephens, 1970a) or electrophoretic separation by charge (Bryan and Wilson, 1971) indicates no specific activity difference between tubules or subunits. A similar conclusion was drawn by Raff, Greenhouse, Gross, and Gross (1971) for subunits of the total tubulin fraction from fertilized sea urchin eggs, separated as a vinblastine precipitate from cell homogenates taken at successive time points during development.

TABLE I

Relative tubulin specific activity in virgin versus *regenerated cilia as an approximate measure of synthesized pool size at any given time point, determined from two separate experiments by approximation of area under the tubulin peak in autoradiographic densitometer traces from samples of known specific activity*

Pulse time	Virgin	Regenerate	% of original in regenerate
30–33 hr	1265/1215	1200/1160	95
27–30	1200/1120	1140/1100	96
24–27	800/775	545/515	67
21–24	860/820	625/590	72
18–21	800/760	540/590	66
6–9	815/785	570/530	68
0–3	575/525	445/405	77

Other major components that remain constant prior to and during ciliogenesis have molecular weights of 280,000, 105,000, 88,000, 50,000, 45,000, 38,000, and 32,000. The latter two components vary somewhat in position and quality since the 5% gel system was designed for maximum resolution in the higher molecular weight region. The 105,000 and 50,000 or the 88,000 and 45,000 molecular weight components may be related as monomer to dimer, and thus only two components rather than four may exist. The 32,000 molecular weight material has been tentatively identified as an *adenylate kinase* on the basis of ammonium sulfate fractionation, enzyme determination, and electrophoretic identification of the most active fraction. Regardless of identity of these minor components, it should be obvious that the bulk of the structural proteins, *i.e.*, these plus tubulin and dynein, do not show any differential synthesis during early development. Since they represent at least three-fourths of the total axonemal protein, the observation of near-constant synthesis of "ciliary" proteins prior to and early in ciliogenesis is easily rationalized. It is the initiation of synthesis of *minor* constituents that marks the beginning of ciliary morphogenesis.

(3.) *Proteins apparently synthesized* de novo *at ciliogenesis*—Since it is difficult to determine pre-existing pools for minor ciliary constituents and nearly impossible to detect a very low but constant rate of pre-ciliogenesis synthesis for such components, the term *de novo* must be used here with caution. In the sense of this study, it may only mean that a marked increase takes place. Regardless of terminology, rapid synthesis of such proteins is of obvious morphogenetic significance.

The proteins that are synthesized at a high rate uniquely at ciliogenesis can be best discussed by molecular weight. Six major components with molecular weights of about 250,000, 200,000, 165,000, 130,000, 73,000, and 68,000 can be readily detected, particularly on the 30 hour pulse tracing (Figs. 3–4; marked with stars). The 250,000, 73,000, and 68,000 molecular weight components are detectable on the 24 hour pulse tracing. The 165,000 and 130,000 molecular weight proteins appear in the 27 hour pulse, while the 200,000 molecular weight component is only obvious in the 30 hour pulse tracing. These differences may simply reflect the relative amounts of these constituents in the axoneme rather than any real differential or sequential synthesis; this appears to be the case for the first detectable components at least. Shorter and more frequent pulses coupled with large scale isolation and very accurate quantitation would be needed to prove sequential or differential synthesis; such is beyond the scope and intent of this current qualitative study.

In sea urchin sperm flagellar axonemes, a 165,000 molecular weight protein has been identified as the linkage material connecting the nine outer doublets together and has been named *nexin* (Stephens, 1970b); ciliary axonemes of other species show additional 80,000 and 240,000 molecular weight bands when subjected to similar nexin isolation procedures. It is possible that in this study the components at approximate molecular weights of 73,000, 165,000 and 250,000 may all be the linkage protein nexin, related as monomer to dimer to trimer. Whatever the interpretation, these and the remaining *"de novo"* components, by a process of elimination, must be either spoke or linkage proteins in the basic "9 + 2" structure. It is not illogical to expect that the assembly of an organelle might be initiated by the synthesis of such vital architectural elements.

(4.) *Proteins with decreased synthetic rates*—This category is the most equivocal, since detection is borderline. One major protein band with a molecular weight of about 300,000 is very prominent on the stained gels. It shows a substantial rate of synthesis on all of the early gels but at 24 hours and beyond it is much less detectable. It is unclear whether it actually decreases in synthesis or the background of other proteins has increased relative to it. This component is also likely to be related to radial or circumferential structural elements. Limited synthesis of such a structural component might offer a useful mechanism for limiting either the length or the number of cilia that an embryo forms at any given time.

Regeneration of cilia from previously pulse-labeled and deciliated embryos

Two major ciliary components (a dynein band and tubulin) and many minor ones are made continuously at constant rate, while at least six other structural protein bands are apparently synthesized *de novo* at and during ciliogenesis. The relative specific activities of these components in regenerated cilia from the original deciliated embryos compared to that of the virgin cilia should give some clue as to the relative amount of each of these constitutents that was synthesized during the pulse period. Does the cell initially manufacture only the amount of material

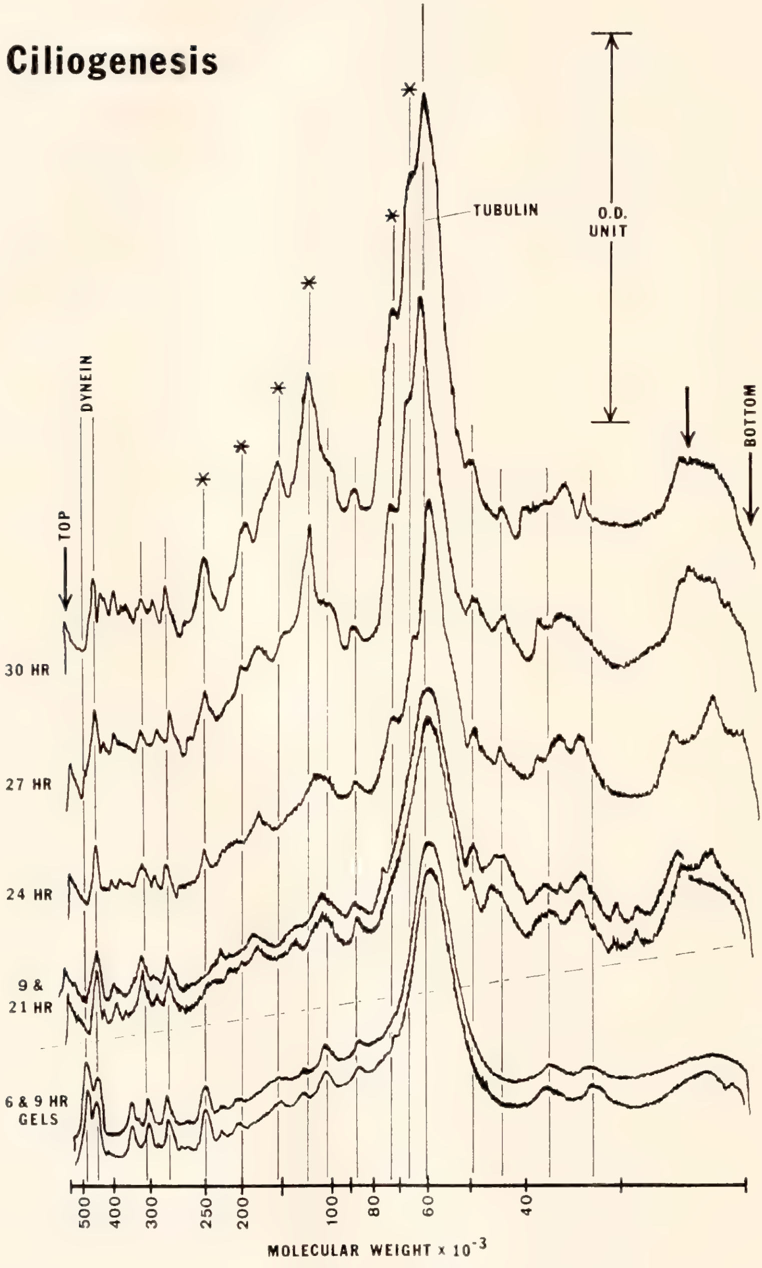

FIGURE 4. Incorporation of ^{14}C-leucine into specific axonemal proteins from embryos labeled at varous times. The microdensitometer tracings were made from gels such as those of Figure 3. The bottom pair of traces are from stained gels of axonemes from 6–9 hour and 9–12 hour pulse periods; the remainder are 9–12 and 21–24 hour pre-ciliogenesis pulse periods, and 24–27 hour, 27–30 hour, and 30–33 hour pulse periods of active ciliogenesis.

that it needs for one generation? Experiments by Auclair and Siegel (1966) indicated that a moderately large precursor pool is available to cilia regenerating at the late gastrula stage, but what is the magnitude of the pool synthesized prior to and during the initial ciliogenesis?

Figure 3G is an autoradiogram of regenerated cilia from an initial 27–30 hour pulse followed by deciliation and should be compared with Figure 3E, the virgin cilia of this same time point. Figure 5 includes representative autoradiographic microdensitometer tracings of regenerated cilia from the same time points illustrated in Figure 4 and includes a superimposed 30–33 hour trace of virgin cilia for comparison.

Microdensitometric analysis of autoradiographic gels from regenerated cilia of previously labeled and deciliated embryos reveal several interesting facts. The dynein band, the general background material in the molecular weight range of 90,000 to 250,000, and the lower molecular weight material below 50,000 have essentially the same specific activity in regenerated cilia as in the original harvest, within an experimental error of ±15%. This could imply that a relatively large and constant pool of these materials has been made prior to deciliation, or simply that whatever has been made will be incorporated into the cilia until the (limited) supply is gone, assuming of course no appreciable synthesis of these components after deciliation. Thus both a large pool with low post-deciliation synthesis or a limited pool utilized totally are consistent with the data.

The tubulin, on the other hand, showed a somewhat decreased specific activity in the regenerates *versus* the virgin cilia in all time points prior to ciliogenesis. In early development, the regenerates have only about three-fourths of the original counts, while the regenerates from embryos pulse-labeled during ciliogenesis have over 95% of the original specific activity (Table I). Assuming that tubulin synthesis continues after deciliation at a rate at least comparable to that during early development, and therefore that most of the tubulin lost to the first harvest is replaced with cold tubulin, the data would indicate a pool three to four times larger than that needed for one generation exists prior to ciliogenesis. This somewhat crude estimate is not unlike that of Auclair and Meismer (1965) who have demonstrated 4 regenerations in the near-absence of protein synthesis. The pool *during* ciliogenesis cannot be estimated accurately by this method since the decrease in specific activity of regenerates *versus* virgin cilia during the active process is within the limits of error; there can be no question that it is even larger, however.

Quite significantly, the *"de novo"* proteins uniquely synthesized during ciliogenesis barely appear in the autoradiograms of the regenerates whose parent embryos were labeled during this period (Fig. 5, top trace). These previously-prominent components are reduced to 10–20% of their original values, a fact indicative of the production of only one "round" of these apparently critical components since essentially all of the protein labeled during ciliogenesis was isolated with the virgin cilia. The striking thing about the autoradiograms of the regenerated cilia is the fact that all pulse points look the same, with the minor exception

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Unmarked arrow indicates the position of the tracking dye front. Asterisks mark the components apparently synthesized *de novo*. The dashed line is an averaged tracing from blank gel-autoradiograms, giving a combined film and instrumental baseline.

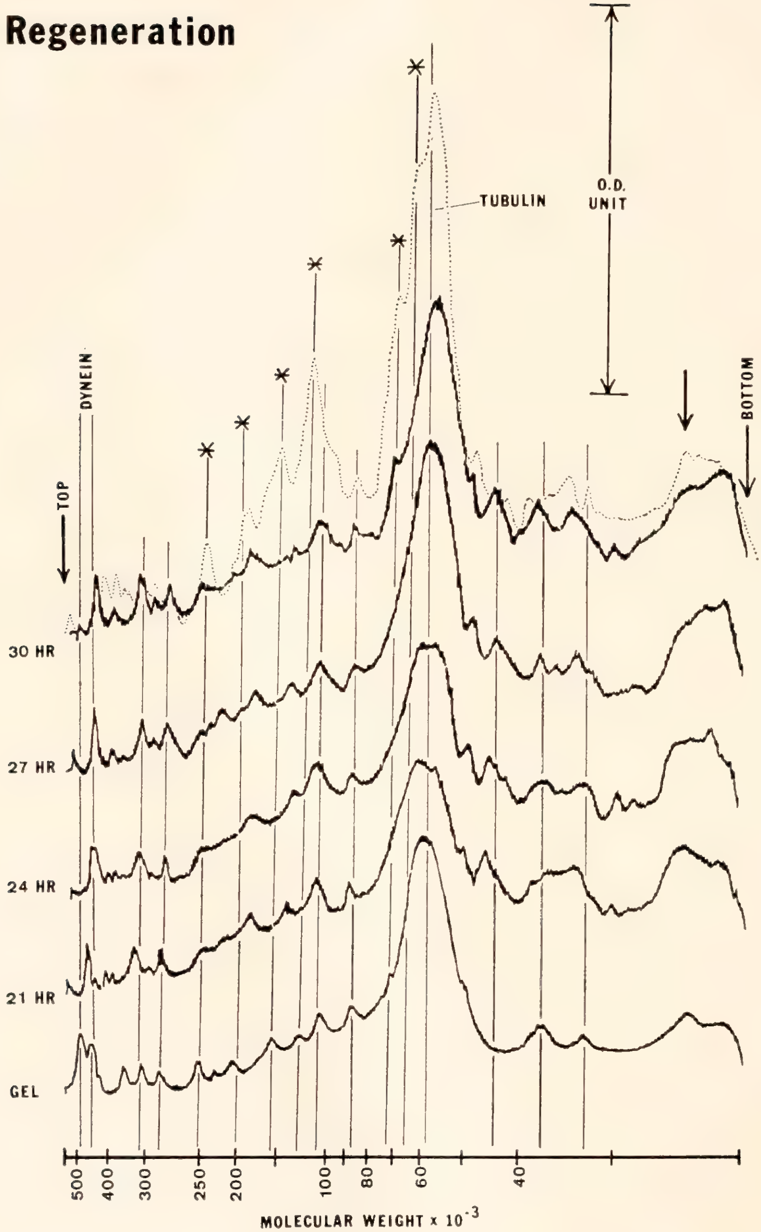

FIGURE 5. Incorporation of labeled proteins into ciliary axonemes upon regeneration from previously labeled and deciliated embryos. Microdensitometer tracings of gel-autoradiograms from regenerated ciliary axonemes. The bottom trace is from a stained gel from a 15–18 hour pulse period. The autoradiogram traces are nearly indistinguishable from one another, except for the higher amount of tubulin label in the 27–30 hour and 30–33 hour regenerates. The dotted trace is the upper trace from Figure 4, permitting direct comparison

of the markedly higher tubulin specific activity during the period of active ciliogenesis.

DISCUSSION

The embryonic construction of a cilium begins well before fertilization with the synthesis of the high molecular weight enzymatic component of dynein. After fertilization the lower molecular weight component is synthesized at a constant rate throughout development. Various "background" proteins are also synthesized in a like manner. All of these components are apparently made well in excess over that needed for one generation of cilia since they have essentially the same specific activity in second generation cilia. Tubulin also shows a constant rate of synthesis up to early ciliogenesis and then increases by about 50%. One protein of molecular weight 300,000 appears to decrease in synthetic rate just prior to ciliogenesis.

Given this large pool of precursor material, some event or events must trigger ciliary morphogenesis at the prescribed moment. Perhaps most important in the scheme of assembly is the apparent *de novo* synthesis of "minor" structural components during active ciliogenesis. These presumably constitute the linkage-spoke complex that gives three-dimensional structure to the axoneme. Nearly all of these *de novo* components are utilized to form the virgin cilia since little label is found in these proteins upon regeneration. The *de novo* synthesis of the linkage complex as an initiating morphogenetic event is not inconsistent with the inhibition studies of Auclair and Siegel (1966); if synthesis of these minor constituents continued at the same rate after the initial ciliogenesis, there would be many "rounds" of these proteins available at the gastrula stage where continued multiple reciliation was demonstrated. Also, "90% inhibition" may still allow such components to be made, especially since ciliary proteins are apparently synthesized preferentially. In contrast to the studies of Auclair and Siegel (1966) and Auclair and Meismer (1965), Child and Apter (1969) demonstrated that growth or regeneration of cilia could be prevented by application of *Pactamycin* just before the scheduled onset of cilia formation. Here 94% inhibition of protein synthesis could be shown. Since protein synthesis was turned off only briefly prior to ciliogenesis, a time when the bulk structural components have already been synthesized, these authors propose that synthesis of a minor protein, critical for ciliary assembly, is inhibited, a conclusion not unlike that drawn from this present study.

The simultaneous operation of post-synthetic events to activate certain structural proteins cannot be ruled out. Mitotic tubulin, for example, can be differentially mobilized from an inactive form during prophase (Stephens, 1972b). It is not inconceivable that phosphorylation of bound GDP, intramolecular disulfide bond formation, or a specific cation addition might serve as "activation" steps, much as these three factors play an important role in the *in vitro* assembly of

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of virgin *versus* regenerated specific activities. The 21–24 hour and 24–27 hour regenerate tubulin is substantially lower than that from comparable time points in Figure 4, indicating roughly a 25% depletion of the labeled tubulin pool upon regeneration. Notation is the same as Figure 4.

flagellar B-tubulin (Stephens, 1971; in preparation). It might be pointed out here that the molecular weights of tubulin obtained from fertilized eggs, isolated mitotic apparatuses, and ciliary axonemes exhibit no obvious numerical differences, indicating that proteolysis is probably not an activation step. Still, specific proteolysis of components other than tubulin offers a reasonable method for mobilizing active structural building blocks from an inactive pool at a discrete time point in development.

One important basic biological question might be raised in light of the above data. If the tubulin and dynein components of the cilium are in abundance well before ciliogenesis and do not require "activation," what prevents them from forming doublet microtubules in conjunction with the mitotic centrioles? An essential morphogenetic event may be the *activation of the centriole*, enabling it to serve as a "crystallization center" for pre-existing and *de novo* components and thus form the basal body of the developing cilium. Perhaps Pickett-Heaps' (1969) argument that participation in mitosis simply ensures the centrioles of equal partitioning in the daughter cells is applicable here; they may be themselves nonfunctional until called upon in later development to serve as basal bodies.

The morphogenetic process in cilia is beginning to show some interesting parallels to other better-studied developmental processes. Sequential gene action, production of "minor" structural components, and subsequent modification of previously-synthesized proteins are well-established events in the morphogenesis of the phage T-4 (*cf.* Wood, Edgar, King, Lielausis and Henninger, 1968). The production of one "round" of *de novo* structural elements brings to mind a similar quantal production of enzymes in the developing cellular slime mold *Dictyostelium* (Newell, Longlands, and Sussman, 1971). Yet many critical questions remain to be answered. Paramount is the issue of the tubulin pool: how large is the pool prior to fertilization, what relative amount of total tubulin is synthesized post-fertilization, how many tubulins are involved at each stage, and into what structures are they incorporated? In addition, how (if at all) is ciliary tubulin "activated" *in vivo?* What is the role of the great excess of high molecular weight dynein component—a pool for ciliary regeneration or a mitotic ATPase? Is the centriole (basal body) unmasked as a "crystallizing center"? What is the nature of the unidentified *de novo* structural proteins and how are they involved in ciliogenesis? How is ciliary length determined? Is there some limiting component mobilized anew at each regeneration? Do micromeres, which never make cilia, synthesize the lower molecular weight dynein band or the *de novo* structural components characteristic of cilia? What is the pattern of ciliary protein synthesis *after* cilia formation and how is it affected by deciliation? Answers to these questions should provide a better understanding of the processes by which a developing embryo gives rise to its first self-generated organelle.

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SUMMARY

1. Cilia were isolated from sea urchin blastula pulse-labeled with ^{14}C-leucine at various time points prior to and during ciliogenesis, the structural components fractionated by SDS-acrylamide gel electrophoresis, and the relative amount of labeled protein determined by autoradiography of gel slices.

2. The two components of the ciliary ATPase dynein are synthesized differentially. The higher molecular weight enzymatic component pre-exists before fertilization; only the lower molecular weight component is synthesized after fertilization and it is made at a constant rate.

3. A number of components with medium and low molecular weights are also synthesized uniformly throughout development. Of these, tubulin alone shows a marked increase in synthesis during late ciliogenesis. This protein is synthesized in three to four-fold excess over that needed for one generation of cilia. All of the others, including dynein, show no significant decrease in specific activity upon regeneration from previously labeled and deciliated embryos.

4. At the initiation of ciliogenesis, at least six minor components appear to arise *de novo* and only in sufficient amount for one generation of cilia.

5. These data support previous findings that the bulk of ciliary protein is made prior to ciliogenesis and in considerable excess, but suggest further that the morphogenetic process is marked by a "round" of *de novo* synthesis of minor but critical structural components.

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SUSPENSION FEEDING BY MARINE INVERTEBRATE LARVAE: CLEARANCE OF PARTICLES BY CILIATED BANDS OF A ROTIFER, PLUTEUS, AND TROCHOPHORE

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The means by which cilia remove particles from suspension are of ecological interest because the clearance mechanism determines how much and what kind of food can be obtained by a suspension feeder in a given environment. Clearance is of physiological interest because cilia which must remove particles from a current of water may require greater strength or more rapid alteration of beat than cilia moving in water alone. When both these aspects are considered, the organization and evolution of the ciliary feeding systems are more readily understood. The entire process of suspension feeding consists of (1) producing a current, (2) filtering or clearing particles from it, (3) transporting the particles to the mouth, and (4) ingesting them (Werner, 1959). Since clearance is the most difficult part of the process to observe, descriptions of feeding mechanisms frequently omit clearance, and discussions of ciliary motion often exclude consideration of the extra load some cilia must bear.

Although many small suspension feeders have the simple arrangement of only one or two ciliary bands, little is known about the manner in which cilia concentrate particles from suspension. There is not the division of labor which Dral (1967) found in the mussel, *Mytilus*, which uses different sets of cilia to function in current production, filtering, and transport of particles. Nor is there the distinct mucus filter found in some other suspension feeders (Werner, 1959). Some authors have suggested mechanisms such as centrifugation (Tattersal and Sheppard, 1934), impingement (Bullivant, 1968a, b), or sedimentation (Davis, 1955), but these cannot account for the high rates at which particles are cleared (Strathmann, 1971). Other authors have felt that contact between cilia and particles is necessary for removing particles from suspension but have been unable to determine just where or how particles are moved relative to the water. Observation is difficult because (1) cilia are small and move rapidly, and (2) water currents, as distinct from the motion of particles, are difficult to observe on this scale.

In this study we used high speed cinefilms of plutei, rotifers, and a trochophore to examine two mechanisms by which particles are removed from suspension by cilia. In one type of suspension feeding a single band of cilia produces a current and retains particles on the upstream side of the band. This type of feeding is exhibited by planktotrophic echinoderm larvae (Strathmann, 1971) and at least some of the tornaria larvae of hemichordates. We will refer to this type as the single band system. In a second type of suspension feeding, one band of cilia produces a current and particles are collected downstream in a groove between this band and a parallel band of shorter cilia. We will refer to this second type as the opposed band system. It is exhibited by bdelloid and floscularíacean rotifers (Hatschek, 1878; Zelinka, 1886; Remane, 1929, 1932), the trochophore larvae of annelids (Hatschek, 1878; Wilson, 1932) and probably also the trochophores of echiuroids (Hatschek, 1880), the veliger larvae of bivalves and gastropod mollusks (Yonge, 1926, Werner, 1955; Thompson, 1959; Fretter, 1967), and entoproct larvae (Jägersten, 1964; Mariscal, 1965).

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We shall try to give a satisfactory account of the mechanisms by which particles are cleared from a suspension in these two systems. We shall then discuss the variability of the two mechanisms and indicate how their limitations may be related to differences in the size of particles which can be eaten and differences in clearance rates (volume of water processed per unit time).

MATERIALS AND METHODS

In California we reared from egg and sperm the plutei of the sea urchin *Lytechinus anamesus* (Echinodermata, Echinoidea) and trochophores of the serpulid worm *Spirabranchus spinosus* (Annelida, Polychaeta). We used methods described by Costello, Davidson, Eggers, Fox, and Henley (1957). We obtained mitraria larvae, gastropod veligers, and tornaria larvae from the plankton near Catalina Island. Bdelloid rotifers resembling *Philodina* were obtained from a hay infusion culture of *Paramecium*. In Hawaii we reared tornariae of Ptychordera flava (Hemichordata, Enteropneusta) from egg and sperm, and we used veligers of *Charonia tritonis* (Mollusca, Gastropoda) newly hatched from egg capsules.

Plutei, a trochophore, and rotifers were filmed on "Plus X" or "4X" film (Kodak) at 100 to 200 frames per second with a Redlake Locam camera with phase contrast or Nomarski interference microscopes (Zeiss). These cinefilms included a time marker for noting rates, all of which were measured at room temperature, about 20 to 22° C.

The plutei were pressed by the coverglass just enough to slow them and to orient them for filming. In this way we could film an optical section across the lateral portion of the ciliated band between the postoral and anterolateral arms. We were less successful in orienting and slowing trochophores without interfering with feeding; but the rotifers remained stationary, fed actively, and maintained a suitable position on microscope slides.

Filmed observations were made of a trochophore feeding on *Phaeodactylum tricornutum*, rotifers feeding on plastic spheres of 1.3 or 2.7 μ diameter (Dow Chemical Co.), and plutei feeding on either *Phaeodactylum* or *Dunaliella tertiolecta*, sometimes in combination with plastic spheres. In other feeding observations we used these algae or particles and also *Amphidinium carteri*, *Cricosphaera carterae*, and Sephedex spheres (Pharmacia).

Even with unnaturally high concentrations of particles, there are relatively few contacts per 100 feet filmed between particles and the portion of the ciliated band in focus. With still higher particle concentrations the larvae ceased clearing particles from suspension. This limited the number of particle captures we could record on film.

In even the best filmed sequences, the movement of water could not be directly observed. This would have required a technique for introducing just upstream from the ciliated band a very small (less than 30 μ diameter) discrete volume of water colored by soluble dye and including suspended particles. (Chinese ink and other current markers commonly used in zoological studies are suspensions of particles and not suitable for observing motion of particles relative to water in clearance). The motion of water was therefore inferred from motion of particles and cilia.

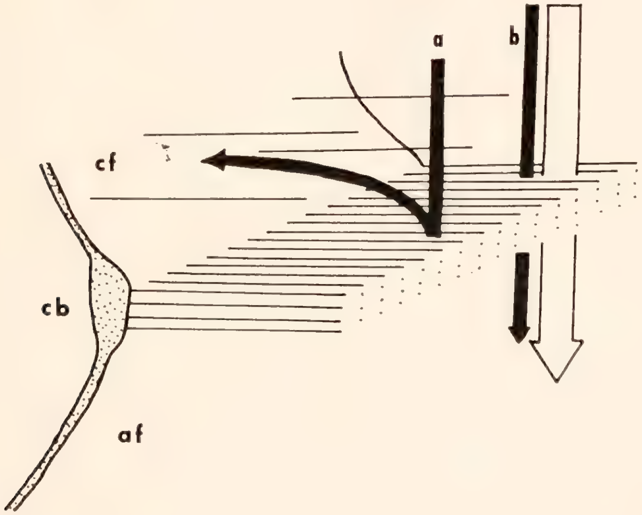

FIGURE 1. Diagram of single band of cilia with upstream collection of particles. For explanation of symbols and abbreviations, see text.

The close array of cilia prevented tracing of individual cilia in successive frames, but positions in a given frame and a composite picture of positions during the beat cycle could be obtained.

RESULTS AND DISCUSSION

Locally induced reversal of beat in the pluteus

The distribution of cilia in the single band system is diagrammed in Figure 1. The band of cilia produces the current (white arrow) used in feeding and locomotion. When a larva is feeding, particles are stopped at the ciliated band and passed back to the cilia of the circumoral field (Fig. 1a), which pass them on towards the mouth. Some echinoderm larvae lack cilia on the circumoral field, and particles are passed along the band of cilia in a series of jumps. When a larva is not feeding, the current is the same but particles are passed through the band of cilia with the water (Fig. 1b). In some forms the cilia of the band can also reverse the direction of the beat and the water current. During a general reversal of beat along the band, the direction of swimming is reversed and feeding ceases or is greatly reduced. All of this can be seen without high speed cinefilms.

The high speed cinefilms of plutei indicate that a locally induced reversal of beat is the clearance mechanism operating at the band. As diagrammed in Figure 2, the cilia have a straight effective stroke and curved recovery stroke. The effective strokes of the cilia of the band sweep a little more than 180°. When the larva is not feeding, particles pass through the band of cilia with the water (Fig. 2), and forward beating continues with no discernible change. In a feeding larva the beat is the same until a particle comes within reach of the cilia. Then an alteration of the beat of several cilia can be seen while the particle slows, changes direction, and is finally pushed back in an arc suggesting a reversed effective stroke of the cilia (Fig. 3a). In some cases the particle is only detained by the brief alteration of beat (Fig. 3b). Because a small particle can pass through the band of cilia close to the position at which a larger particle is being retained, the change of beat which retains a particle must be local.

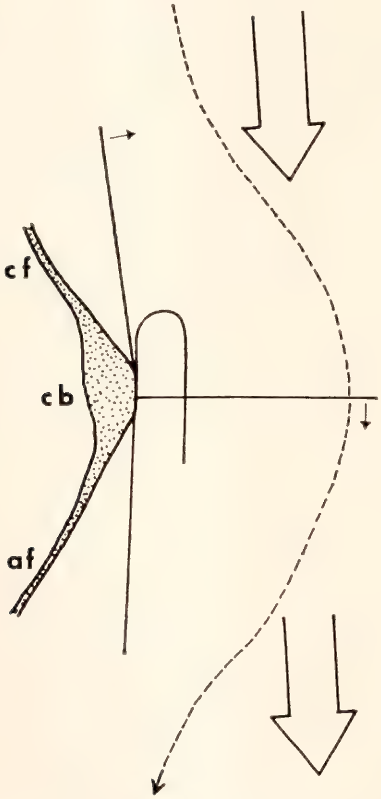

FIGURE 2. Diagram of form of beat of cilia in the pluteus and path of a particle through the band when the pluteus is not feeding. For explanation of symbols and abbreviations, see text.

The change in beat may be triggered by a mechanical disturbance of the cilium, and a larger particle may be a more effective trigger. Small particles (bacteria and polystyrene spheres of 1 or 2 μ diameter) pass through the band of cilia (Fig. 3c) when larger particles (*Dunaliella* or *Phaeodactylum*) are retained. When 1.3 μ polystyrene spheres and cells of *Amphidinium* were present in equal concentrations, the plutei ingested more than five times as many cells of *Amphidinium*.

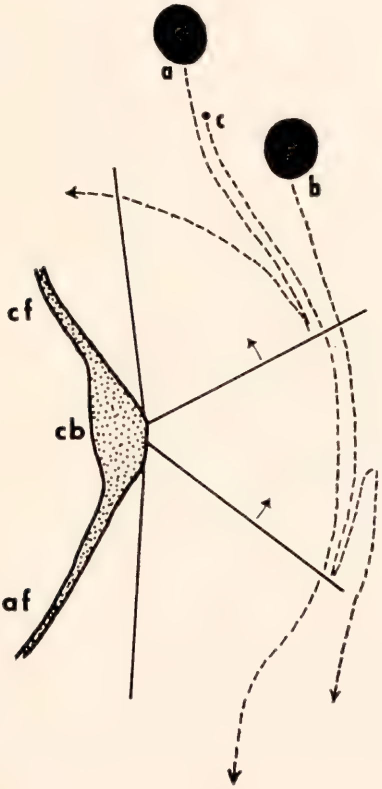

Figure 3. Diagram of paths of particles through the band of cilia when the pluteus is feeding. For explanation of symbols and abbreviations, see text.

The whole process of altering beat and retaining a particle occurs very rapidly. In a feeding larva particles approaching the cilia are moving about 0.07 to 0.11 cm/sec. (Some larvae which were not feeding produced a faster current across the band, but this was not a consistent difference between feeding and non-feeding larvae.) A particle's forward motion is stopped about 0.02 to 0.06 sec after it has come within reach of the cilia (estimated as 25 μ from the base of the cilia). The particles are moved back toward the circumoral field at about 0.06 to 0.10 cm/sec. Forward beat is resumed after about 0.06 to 0.14 sec.

In the sequences in which the cilia retaining a particle were clearly in focus, a reversed recovery stroke could be seen; but in these sequences no other particles were present to indicate a forward beat elsewhere. Thus, we cannot be certain that these reversals were not general reversals coinciding with the arrival of a particle at the band. In the sequences in which alteration of beat was clearly local, the cilia pushing the particle were not as clearly in focus; and we could only deduce from the motion of the particle that the alteration of beat included a reversed effective stroke. Though an induced local reversal of the effective stroke could not be directly observed, we can find no other interpretation consistent with our observations.

That a reversal remains localized is not surprising, since there is only a single cilium per cell in echinoderm larvae. It is more difficult to account for the rapidity of the reversal. A particle carried within reach of the cilia is moving almost as fast as the cilia in their forward effective strokes. The cilia must be stimulated during the beginning of their forward effective stroke, and either these cilia or their neighbors must reverse beat before the forward stroke is completed. The current velocities, timing, and particle motion indicate that this very rapid reversal of beat does occur within a fraction of the time it would take to complete a forward stroke.

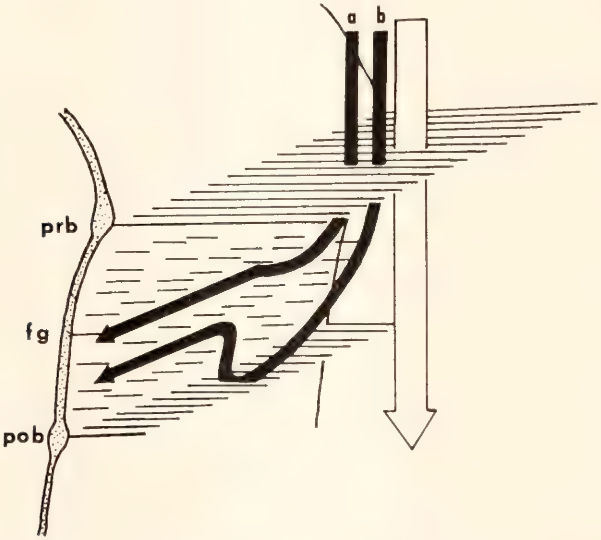

FIGURE 4. Diagram of two opposed bands of cilia with collection of particles in a food groove. For explanation of symbols and abbreviations, see text.

Opposed band system of bdelloid rotifer and trochophore

The distribution of cilia in the opposed band system is diagrammed in Figure 4. Previous investigators agree that the long cilia of the preoral band produce the major current used in feeding and locomotion, and that the short, fine cilia of the food groove convey the collected particles along the groove to the mouth, but they variously describe particles as being brought directly into the food groove by the long cilia of the preoral band (or trochus or prototroch) (Fig. 4a) or as being first carried to the shorter cilia of the postoral band (or cingulum or metatroch) (Fig. 4b) which then convey them to the food groove. High speed cinefilms of bdelloid rotifer and trochophore and observations of rejection mechanisms of larvae indicate that both preoral and postoral bands are essential to clearance. We have concluded that the opposed beat of cilia of the preoral and postoral bands results in increased movement of the preoral cilia relative to the water in the latter half of their effective stroke and that most of the clearance of particles from water occurs at this point.

The cinefilms of bdelloid rotifers indicate that the cilia of the preoral and postoral bands beat towards each other (Fig. 5) with an approximately straight effective stroke and curved return stroke. Particles enter in the major current (Fig. 5A, upper arrow). Most of the particles within reach of the preoral cilia are swept into the food groove (Fig. 5a). A few particles observed posterior to the preoral cilia indicate a current in this region (Fig. 5A, lower arrow), but this water has been largely cleared of particles. Many of the particles swept into the food groove never come within reach of the postoral cilia (Fig. 5a). Particles just beyond the reach of the preoral cilia continue to move posteriorly (Fig. 5b). For clearance to occur, the cilia of the preoral band must push the particles relative to the water; this probably happens in the latter half of the effective stroke as the particles are carried into the food groove. Mucus could also aid in clearance; if the cilia of the preoral band were to pick up mucus as they slide past the food groove on the recovery stroke, then they might hold particles more firmly on their effective stroke. Particles in the food groove can be carried toward the base of the cilia of the preoral band by the return stroke (Fig. 5e). This action of the return stroke may help keep particles in the food groove.

Then what is the role of the postoral band of cilia? Although these cilia may help retain particles in the food groove or serve to catch particles slipping beyond the tips of the cilia of the preoral band (Fig. 5c), they also appear to play a more direct role in the clearance mechanism. In some instances particles which have slipped beyond reach of the cilia of the preoral band are also carried beyond the postoral band of cilia, only to be conveyed anteriorly into the food groove (Fig. 5d). Since many of these particles are clearly beyond reach of the cilia of the postoral band, they must be carried in currents (Fig. 5d) presumably produced by the postoral band of cilia. Since the two bands of cilia are producing currents in opposite directions, water must be pushed away from the food groove where the cilia are completing their effective stroke. This water movement would keep most particles from slipping beyond the reach of the cilia of the preoral band and would increase the movement of preoral cilia and particles relative to the water.

Observations of serpulid trochophores showed that the cilia of the two bands beat in the same manner as the cilia in the rotifers. Particles coming within reach of the cilia of the preoral band are carried into the food groove. The orientation of the filmed trochophore relative to the camera prevented detailed analysis of particle paths near the postoral band of cilia, but since the distribution and beat of the cilia and the paths of particles are very similar, it seems likely that trochophores employ the same mechanism of clearance as bdelloid rotifers.

However, in the serpulid trochophores, regulation of clearance may be different. The rotifers cleared particles continuously when the wheel organ was extended, although the collected particles were often rejected at the mouth. The trochophores could similarly reject particles at the mouth, passing them down the neurotroch; but they could also stop filtering while continuing to swim. While the preoral band of cilia and cilia of the food groove stopped beating, particles were not collected behind the preoral band of cilia but were carried posteriorly with the water. This supports our view that the function of the trochophore's postoral cilia in clearance

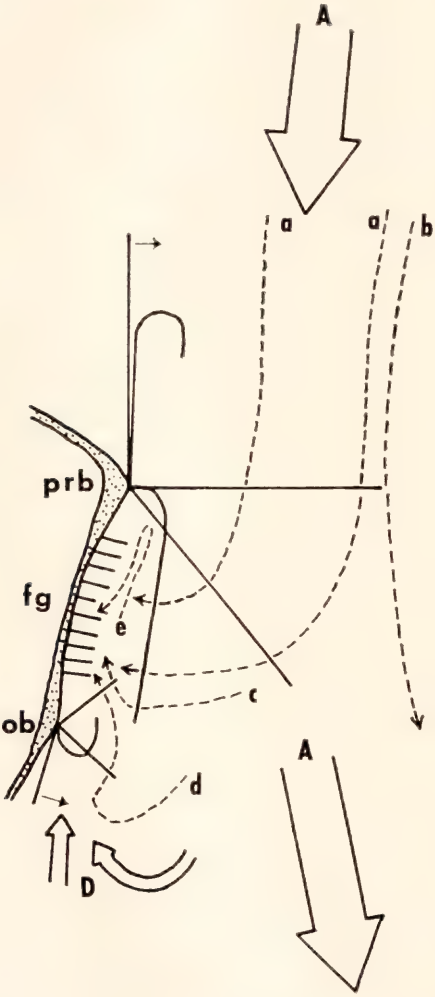

FIGURE 5. Diagram of direction of beat of cilia, currents, and particle paths in a bdelloid rotifer. Direction of ciliary beat, currents, and particle motion are similar in the trochophore. For explanation of symbols and abbreviations, see text.

is the same as in the bdelloid rotifer. A mitraria larva and gastropod veligers collected from the plankton also could continue swimming without clearing particles, presumably again by stopping beat of the postoral cilia.

Occurrence of the two systems

The single band system has been found in all planktotrophic echinoderm larvae studied (Strathmann, 1971) and also occurs in the tornaria larva of hemichordates. In the tornariae which we have observed, the ciliated bands beat away from a ciliated circumoral field, and particle movement in feeding and rejection is like that in an echinopluteus or bipinnaria. These tornariae included individuals having no tentacles throughout development and early stage larvae of *Ptychodera flava*, which appeared to be developing tentacles. Garstang (1939) described a very different mode of feeding for tentaculate tornaria larvae. In Garstang's tornariae, particles first contact the aboral field and are then moved across the band onto an unciliated circumoral field. No mechanism is offered by Garstang for concentrating particles until after they have entered the mouth. It seems unlikely that the larger tentaculate stages should have such a different feeding mechanism, but further studies of tentaculate forms would be of interest.

The single band clearance mechanism, with an induced localized reversal of beat, could conceivably occur in other animals whose mechanism of clearing particles has not been satisfactorily explained. For example, the lateral and frontal cilia of some brachiopods might function much as the ciliated band and cilia of the circumoral field of the tornaria and echinoderm larvae. During feeding, the direction of ciliary beat, currents, and the movement of particles down the tentacles or filaments are quite similar (Atkins, 1956). Some brachiopods have one cilium per cell (Atkins, 1958). An induced local reversal of beat of the lateral cilia could explain how small particles are retained on the frontal surface of the filaments. But if other lophophorates were found to exhibit the single band system then we would have to alter our opinions on its limitations, for in some lophophorates there are several cilia per cell (figures in Hyman, 1959), and the lateral cilia run to somewhat greater length than the cilia of the ciliated band in tornaria and echinoderm larvae. More detailed observations of the lateral cilia of lophophorates during feeding and rejection should indicate the extent to which an induced local reversal mechanism is employed in this group. If this mechanism does occur, there should be a break in the metachronal wave when a particle is retained by the lateral cilia.

The opposed band system appears to occur in bdelloid and floscularíacean rotifers, the trochophores of echiuroids and annelids, the veligers of mollusks, and in entoproct larvae (for references see above). However, some authors have not included a postoral band of cilia when describing larvae of mollusks, annelids, or echiuroids which feed and have a well-developed preoral band. In many cases this omission is undoubtedly an oversight. The shorter cilia of the postoral band could easily be confused with the cilia of the food groove, particularly when the constituent simple cilia making up a compound cilium have separated from each other during fixation. However, Newby (1940) states that the postoral band is absent in the planktotrophic larva of *Urechis caupo*, while noting its presence in other echiuroid larvae. Either he is mistaken, or there are larvae which superficially resemble the ones we have considered but employ a very different mode of feeding. Is it possible that a single band of cilia could concentrate suspended particles, collecting them on the downstream side? The cinefilms of the pluteus with polystyrene spheres showed that occasionally a small particle is temporarily caught downstream from a single band of cilia by being repeatedly pulled back toward the band by the return stroke. But it is difficult to imagine how such a system could achieve the clearance rates which seem to be necessary in marine larvae. If particles were to adhere with sufficient tightness to the preoral cilia, how would they be transferred to the cilia of the food groove?

The occurrence of one of these two clearance mechanisms in an animal does not mean that other clearance or feeding mechanisms are not also present. In the tornaria and bipinnaria larvae, particles can move onto the heavily ciliated circumoral field and travel along it for some distance without contacting the ciliated band. This means that the cilia or mucus on the circumoral field function to some degree in removing particles from the current produced by the ciliated band. Cilia on the circumoral field of echinoplutei may function in clearance also. Multiple feeding mechanisms may occur in trochophores. Thorson (1946) describes small trochophores with very large diatoms in their digestive tracts. These diatoms could not have been captured by the opposed band system that we have described, so another or supplementary mode of feeding must be employed by these forms. We must therefore exercise some caution in moving from a description of clearance mechanisms of ciliated bands to inferences concerning the feeding biology of the whole animal, and it will be understood that subsequent remarks apply only to clearance by the bands in the single and opposed band systems.

Size of particles cleared from suspension

The efficiency with which a particle is cleared from suspension will vary with its size, shape, and other properties, but we can see in a general way how the single and opposed band systems will differ as to the size of particles cleared. In the opposed band system the preoral and postoral bands must be close enough for the opposed action of the bands to be effective, and the size of the particles which can be collected and transported in the food groove is accordingly limited (see also Werner, 1955 and Fretter, 1967). In the single band system the circumoral field is broader, and because its curvature helps retain particles of diameter greater than the length of the cilia, the upper limit on size is less obvious. Fully developed echinoderm larvae ingest Sephadex spheres of 60 to 80 μ diameter (Strathmann, 1971) and the tornaria larvae ingest spheres over 100 μ in diameter. Garstang (1939) cites the observation of a polychaete larva passing along the circumoral field into the mouth of a tornaria.

In the opposed band system there is no obvious limit to clearance at the small end of the size range. Very small particles might not slip between cilia if held by mucus, and the bdelloid rotifer does, in fact, eat 1.3 μ diameter spheres. In the single band system in the pluteus, we did not see the 1.3 μ diameter spheres trigger a local reversal of beat and they were eaten at a lower rate than the larger cells of *Amphidinium carteri.* It seems likely that small particles cannot be cleared as efficiently by the single band system because they do not trigger a reversal of beat. Alternative possibilities are (1) that the relative inefficiency is clearing polystyrene spheres from suspension is due to a property other than size and (2) that the plutei of *Lytechinus anamesus* cannot be taken as representative of all animals with the single band system.

Components of the clearance rate

From the description of the clearance mechanisms we can see that clearance rates might be estimated from an equation such as

$$C = 1_b(1_c - 1_r)v$$

where 1_b is the length of the ciliated band, 1_c the length of the cilium, 1_r a correction factor for the return stroke (where the cilium is not capturing particles), and v is the velocity at which the particles are carried within the capture distance, $1_c - 1_r$. We should therefore be able to relate clearance rate to morphological features and analyze its components; and we should also be able to calculate clearance rates using v from the cinefilms and measurements of cilia and band length.

In the bdelloid rotifer, particles passing within 12 μ of the base of the preoral cilium were almost always conveyed into the food groove. When the cilium was about half way through the effective stroke, these particles were moving at 0.08 to 0.13 cm/sec relative to the rotifer. Particles passing closer to the tip of the cilium, 12 to 15 μ out from the base, moved at speeds up to 0.18 cm/sec but occasionally slipped beyond the cilia and were lost. Without data on the frequency with which particles passed a given point and were captured, we must content ourselves with a rough approximation and say that for the rotifer clearance will be on the order of 10 × 0.1 cm/sec or about 0.9 ml/day per mm of ciliated band.

For the pluteus we can calculate a clearance rate per unit length of band as about 20 × 0.1 cm/sec or 1.7 ml/day per mm of ciliated band. Clearance rates estimated from ingestion rates for other echinoderm larvae agree with this calculated value within a factor of two. This is good agreement considering the errors which enter into both types of calculations.

Though data for v are scant and difficult to obtain, data on the length of cilia and length of band is easily acquired. We can relate variations in morphology to differences in clearance rate and see that different clearance mechanisms will evolve differently in response to the same factors.

Variability in the two systems

The animals known to have the single band system exhibit little variability. In the echinoderm larvae the cilia of the band are about 25 μ long. The cilia are quite fine and appear to be simple rather than compound. Cilia of the band of the tornaria larvae are of similar length and appearance to those of echinoderm larvae and are probably simple cilia also. Compound cilia are present in the telotroch of tornariae, but these function in locomotion, not feeding. In all these larvae the ciliated band is long and convoluted increasing greatly during development. Within the echinoderm larvae there is some variation in band width, but clearance rate is approximately proportional to band length (Strathmann, 1971).

In the opposed band system the preoral cilia are about 15 μ long in some bdelloid rotifers and range up to 100 μ long in some prosobranch veligers. In some species the band length increases greatly as the animal develops. In others, there is relatively little increase. Thus, the opposed band system exhibits much greater variation than the single band system. This greater variability in the opposed band system may be related to the presence of compound cilia in the preoral and postoral bands, whereas the single band system appears to be limited to simple cilia, though the reasons for this limitation are at present unknown.

Harris (1961) argued that single cilia all have about the same bending couple, and therefore the velocity of a cilium relative to the water can be related in an approximate manner to the length and distribution of cilia. Thus the velocity decreases with the length of the cilium and increases with the number of cilia in a compound cilium and with the number of cilia across the band. It follows that in compound cilia the velocity of beat and length of cilium can be varied somewhat independently. This must give an extra dimension in the design of a larva having compound cilia. For example, with a band of simple cilia, increasing the length of the cilia might permit clearance of larger particles from suspension but at the expense of lowering the velocity of beat and hence the clearance rate. With a band of compound cilia the number of constituent cilia could be increased as the length of a compound cilium is increased, so that larger particles could be cleared without any lowering of the clearance rate. Moreover, with compound cilia it may be possible to increase clearance rate per unit length of ciliated band by increasing both velocity of beat and length of cilium. With simple cilia a limited increase in clearance rate might be achieved by increasing the number of cilia per unit length of band, but clearance rate must be largely proportional to band length.

Variation is also found in less obvious features of the opposed band system: the number of rows of cilia in the preoral band (see Fretter, 1967 on veligers) and the relative lengths of cilia in each of these rows (see Hatschek, 1878, 1880, 1885 on trochophores). The trochophore of *Spirabranchus* has three rows of preoral cilia with the cilia of the middle row much longer than those of the other two rows. The length of the preoral cilia relative to the width of the food groove varies, as do the relative lengths of preoral and postoral cilia. In the rotifer studied here, the preoral cilia were about 12 to 16 μ long, the postoral cilia about 4 to 5 μ; in the trochophore the longer preoral cilia were 30 to 40 μ long, the postoral cilia 13 to 17 μ; and in the newly hatched veliger of *Charonia tritonis* preoral cilia are about 100 μ, the postoral cilia about 20 μ. The effect of these less obvious differences on the clearance mechanism and the clearance rates with variously sized and shaped particles is not known.

Limitations inherent in the single band system

The single band system appears to be limited to simple cilia and in all known cases, to one cilium per cell. Yet one might expect that compound cilia would permit a greater clearance rate per unit length of band by increased velocity of beat or increased length of cilia. Since compound cilia are present in the telotroch of the tornaria larva, their absence in the band must be related to the requirements of the clearance mechanism. Perhaps an induced local reversal of beat cannot be managed with sufficient rapidity by compound cilia. Even with simple cilia, the velocity of ciliary beat in a feeding larva may be limited by the speed with which a reversal can occur.

Given the restriction to simple cilia, two other possible limitations are the maximum bending moment or torque and the stiffness of a cilium. Our lack of knowledge of cilia and the complexity of the movement of water, particles, and cilia prevents an accurate calculation of the bending couple or stiffness exhibited by this system. In addition, the particle motion relative to the larva can be measured, but motion relative to the water is less certain and could vary between 0 and 0.2 cm/sec. Several cilia alter their beat when a particle is retained. With several cilia pushing a particle or moving a sizeable parcel of water along with it, the required bending moment or stiffness would be greatly reduced. Nevertheless, by accepting several assumptions and approximations, we can examine the nature of these limitations in terms of a simplified model.

First, what bending moment might be required for a cilium to push a particle of 10 μ diameter back toward the circumoral field? Referring to Stoke's law, even though all the assumed conditions may not be met, we can calculate the drag force on the particle as $F = 3\pi\eta DV$, where η is the viscosity of water, D the diameter of a spherical particle, and v the velocity with which the particle is pushed relative to the water. The cinefilms show that v may be on the order of 0.1 cm/sec, which means that the drag force is therefore about 10^{-5} dyne. If the particle is 20 μ from the base of the cilium, the bending moment is about 2×10^{-8} dyne-cm. Yoneda (1960) found that a component cilium of the abfrontal cilium in *Mytilus* can exert a torque of 2×10^{-8} dyne-cm while resisting a glass needle. Brokaw (1966) calculated bending couples one order of magnitude smaller for sperm tails swimming in a viscous medium.

If the cilium were to bend too much while pushing the particle through the water, it would slip past the particle. Assuming that the cilium in the above example cannot be deflected more than 4 μ without losing the particle, we can calculate the required stiffness as $S = Ml^2/2d$, where M is the bending moment, l the distance from the base of the cilium, and d the deflection at this distance. Then the stiffness is 10^{-10} dyne-cm^2, which is three to four orders of magnitude greater than the stiffness calculated for two kinds of cilia and a sperm flagellum (Rikmenspoel and Sleigh, 1970). Either these calculations are very inaccurate, or these cilia do have a much greater stiffness, as their job might require. Since this stiffness implies that the elastic moduli of ciliary components are greater than have been found for other biologic materials (Brokaw, 1966), it seems more likely that the model is inappropriate for an exact calculation of the required bending couple or stiffness.

The three possible limitations discussed above would affect the length of the cilium or the velocity of the particle movement, and thus only the clearance rate per unit length of band. An increase in the length of the ciliated band would increase the clearance rate but at some cost to the animal. For example, if we imagine that a larva is initially to have a longer band, it must develop from a larger egg, and then fewer eggs can be produced. Other compromises could involve the requirements of swimming or escape responses, and may be too complex and varied for analysis to be profitable; but we can begin to see how the limitations of cilia ultimately restrict the evolutionary strategies of ciliary suspension feeders by limiting clearance rate per unit length of band, and as knowledge of cilia increases, so will our insight into the organization and activities of these animals.

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SUMMARY

1. High speed cinefilms of a serpulid trochophore, a bdelloid rotifer, and an echinopluteus show two ways cilia remove particles from suspensions.

2. In the pluteus a particle passing through the band of cilia triggers a localized change of beat which appears to be a reversal and which retains the particle on the upstream side of the band of cilia. Retention of particles by an induced local reversal of beat implies that the stimulus occurs during the forward effective stroke of a cilium and that the reversed effective stroke of this cilium or its neighbors begins before the forward stroke is completed. For this system, clearance and transport of particles, rejection of particles, and swimming can be accomplished by a single band of cilia.

3. In the bdelloid rotifer, and probably in the serpulid trochophore, the opposed action of parallel preoral and postoral bands of cilia apparently causes the longer preoral cilia to push particles relative to the water during the latter part of the effective stroke. This system has the preoral band of cilia function in clearance and swimming, the postoral band in clearance and rejection, the food groove cilia in transport of particles.

4. These two clearance mechanisms may be of wide distribution. Echinoderm larvae and the tornariae of hemichrodates appear to employ the induced local reversal of beat. The trochophores of annelids and echiuroids, veligers of mollusks, entoproct larvae, and bdelloid and flosculariacean rotifers appear to employ the system with two opposed bands of cilia.

5. Clearance rates can be estimated from velocity of particle movement, length of cilia, and length of ciliated band.

6. Variations in ciliation in these two feeding systems are discussed in terms of differences in clearance rates and the size range of particles cleared from suspension.

7. Factors which may inherently limit clearance rate are also discussed.

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THE EFFECT OF DELAYED FERTILIZATION IN SOME SPECIES OF THE GENUS *TISBE* (COPEPODA, HARPACTICOIDA)

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Sex-determination in Copepods appears to be rather complex and in most cases still obscure. Two types may, however, be clearly distinguished, namely, environmental and genetic determination of sex.

Takeda (1950) and Egami (1951) reported that external conditions, such as chemical agents and temperature, were sex controlling factors for the harpacticoid copepod, *Tigriopus japonicus.* A similar influence of temperature was observed by Battaglia (1959a) in natural populations of *Porcellidium.*

Later a polygenic control of the sex-ratio was proposed for *Tigriopus* (Ar-Rushdi, 1958).

In copepods of the genus *Tisbe* experiments of inbreeding and selection suggested a genetic determination of sex. In fact, it was found that in *T. gracilis* and *T. reticulata* inbreeding produces a strong increase of males and that selection towards males is always effective, but apparently ineffective towards females (Battaglia, 1958; Battaglia and Malesani, 1959; 1962). On the basis of these and other observations a hypothesis was advanced that in *Tisbe* sex is determined by a multifactorial system consisting of several dominant factors for femaleness (F) and several recessive factors for maleness (m), not allelic and spread over various chromosomes. The full expression of factors F appears to be conditioned by their heterozygous state, whereas the maleness factors would express themselves only in the recessive homozygotes mm. In other words, the degree of femaleness appears to be a function of the number of heterozygous loci F and m; the degree of maleness would instead be a function of the degree of homozygosity of both kinds of loci.

This hypothesis—sex under the control of a multifactorial system—leads, moreover, to an understanding of the variable sex-ratio in these animals including families with a strong excess of males or of females (Battaglia, 1958; Battaglia and Malesani, 1959). Their distribution is, however, in most cases close to normal.

The above interpretation infers that only internal conditions—that is consanguinity—appear so far to affect the sex-ratio in *Tisbe*, where a higher proportion of males is a function of homozygosity.

Some observations made incidentally during recent investigations on two species of *Tisbe* (Volkmann-Rocco and Battaglia, 1971) suggest that the sex-ratio may be affected in these species by an external factor, delayed fertilization. Such an influence of the overmaturation of eggs on the sex-ratio has been proved especially in amphibians and butterflies. Thus, a series of detailed investigations was carried out, in order to obtain a wider knowledge on how a delay in fertilization might influence sex-ratio in *Tisbe*.

MATERIAL AND METHODS

The experiments were carried out at constant temperature of 18° C and salinity of about 36‰. The material consisted of 3 species of *Tisbe: T. dobzhanskii* Volkmann-Rocco and Battaglia, collected in Anzio (Italy) in June 1968; *T. holothuriae* Humes from the Island Ponza (October 1969); *T. clodiensis* Battaglia and Fava, one population from the Lagoon of Venice (August 1967) and the other from the Island Ponza (October 1969). About 30 ovigerous females per species were chosen at random from cultures kept in the laboratory under standard conditions, and isolated in single dishes containing 20 cc of filtered sea water and 0.5 cc of a suspension of *Dunaliella* or *Platymonas*. After deposition of the egg-sac the females were removed and a piece of boiled wheat was added as food for the freshly hatched nauplii, according to the technique described by Battaglia (1959). The offspring were checked once every 2 days. They were used partly for the experimental and partly for the control series. When the 4th copepodite stage was reached, control pairs were set up by putting together one female copepodite and one male from different families, while the individuals which later should provide the experimental pairs were kept isolated in order to prevent them from mating, and thus to delay fertilization of the females.

Since the life cycle differs more or less from species to species, the control pairs were set up at different times, namely 6 days after hatching for *T. holothuriae*, 7 days after hatching for *T. dobzhanskii* and *T. clodiensis* from Venice, and 8 days after hatching for *T. clodiensis* from Ponza. Each dish was checked once every day in order to detect mating and to discard pairs of the same sex. There is, in fact, a possibility of putting together 2 female or 2 male copepodites, since sexes may be distinguished only with difficulty at the 4th copepodite stage. When the female became ovigerous, she was transferred to a freshly prepared dish to guarantee the most favorable environment for the larvae. As soon as these hatched the female was removed and the larvae were reared as described before.

When part of the control females had become ovigerous, males and females of the experimental series, until then kept in isolation, were put together, one pair per dish. The introduction of the male was delayed as long as possible depending on the species. It was then observed if mating occurred and if the female produced an egg-sac and viable offspring. The ovigerous females were transferred to a new dish, as described for the controls, and removed soon after the deposition of an egg-sac. When the offspring had become adult, they were counted and sexed; the same procedure was adopted for the offspring of the controls.

The control series will be referred to as sample C, the experimental series as sample E.

A statistical analysis and comparison of the mean sex-ratios of samples C and E was carried out with the chi-square test. However, this was not considered sufficient, because of the heterogeneity of the sex-ratio data. In order to exclude heterogeneity from the calculations and to have a better statistical evaluation of the differences between control and experimental series, only the families significantly shifted in favor of males were taken in consideration and compared with the χ^2-test.

Significance of the sex-ratio of each family was calculated by individual chi-square test. Because of the complex and still obscure mechanisms of sex-determination in *Tisbe*, the expected frequencies could not be based on an extrinsic hypothesis. Thus, the null hypothesis has been extracted from the control series.

For *T. dobzhanskii* a ratio of 42% females was taken as null hypothesis, a value which agrees well with previous observations (Volkmann-Rocco and Battaglia, 1971).

For *T. clodiensis* from Venice a sex-ratio of 47.5% females was used as expected value coinciding rather well with observations made by Lazzaretto-Colombera and Polo (1969) and by Fava (Istituto di Biologia del Mare, CNR, Venezia personal communication).

For *T. clodiensis* from Ponza the ratio used in the null hypothesis was 41% females, and for *T. holothuriae*, 45%.

Families containing less that 20 individuals were discarded to exclude errors due to differenial mortality, the number of these families was however low and is reported later.

RESULTS

Tisbe dobzhanskii *(from Anzio)*

In *T. dobzhanskii* from Anzio the average time between hatching of the nauplii and egg-sac production was observed to be 10–11 days, thus confirming recent findings by Volkmann-Rocco and Battaglia (1971).

Thirteen days after hatching, when the females of the controls had become ovigerous and in part had already given offspring, the isolated males and females were put together, one pair for each dish. Within the first 20 minutes many of the 46 pairs were found copulating. In all cases the females extruded an egg-sac on the following day, that is, 14 days after hatching. Males and females of the experiment had been kept isolated for 6 days, thus mating was delayed for about 5 days and egg-sac production for 3–4 days compared with the controls.

From 40 control pairs 3 females produced an egg-sac which did not develop; one family containing very few individuals was discarded. The sex-ratio of the offspring of the remaining 36 pairs was calculated to be 41.12% ♀♀. The average number of females and males per family were 19.9 and 28.5, respectively. These values are, however, only suggestive. As we see from Table I there is a great heterogeneity in the various families. In some families a strong shifting of the sex-ratio in favor of males or females was observed; namely, in about 27% of the families it was significantly shifted in favor of males; in about 22% in favor of females (Table I).

From the 46 females kept in isolation for 13 days, 3 produced an egg-sac which failed to hatch; one was discarded because of the low number of offspring. From the remaining 42 families, 421 females and 1841 males were obtained, corresponding to a sex-ratio of 18.61% ♀♀. Differential mortality may be excluded, since the average number of offspring per female (53.8 individuals) did not drop, but slightly increased. A comparison between this value and the sex-ratio of the controls yielded a highly significant value of the chi-square with $P < 0.001$ (Table II). Although, as mentioned above, the progeny were not homogeneous, a comparison could be easily carried out between samples C and E by comparing the numbers of teligenous and arrhenogenous families. In sample E, 73.8% of the families had a sex-ratio which was statistically significant in favor of males (Table I). A comparison with the 27.7% of arrhenogenous families of sample C yielded $\chi^2 = 16.483$ ($P < 0.001$).

T. clodiensis *(from Venice)*

For *T. clodiensis* from the Lagoon of Venice the time between hatching of the nauplii and egg-sac production was found to be 12–13 days coinciding with the results obtained by Lazzaretto-Colombera and Polo (1969).

TABLE I

Percentages of families with a sex-ratio (A) not significant by individual χ^2 test, (B) significantly shifted towards females, and (C) significantly shifted towards males without (Control) and after artificially delayed fertilization (Experiment)

		Control			Experiment		
		% Families	Range % ♀♀	Mean % ♀♀	% Families	Range % ♀♀	Mean % ♀♀
T. dobzhanskii from Anzio	A	50.00	29.8–55.8	41.1	19.09	29.4–50.6	40.0
C = 36 families	B	22.23	58.8–73.3	66.3	7.10	58.1–66.6	62.0
E = 42 families	C	27.77	17.0–28.1	22.3	73.80	0.0–26.0	9.4
T. clodiensis from Venice	A	65.85	34.3–58.8	47.3	58.92	33.3–59.2	43.2
C = 41 families	B	19.51	62.7–90.0	73.5	8.92	62.7–70.0	77.4
E = 56 families	C	14,63	0.0–25.8	11.9	32.14	0.0–31.2	17.4
T. clodiensis from Ponza	A	55.17	27.7–50.0	39.1	38.77	29.0–53.0	38.38
C = 29 families	B	24.13	55.3–83.3	62.9	14.28	53.3–82.4	62.7
E = 49 families	C	20.68	9.0–24.4	17.6	46.93	1.8–27.3	14.0
T. holothuriae from Ponza	A	37.93	31.1–55.1	43.8	36.95	33.3–55.5	43.5
C = 29 families	B	37.93	60.8–84.7	68.8	19.56	62.5–95.1	72.3
E = 46 families	C	24.13	10.2–29.6	19.5	43.47	0.0–30.1	13.1

Since the isolated non-fertilized females were full of eggs which hardly seemed to be retained in the oviducts, the experimental pairs were set up 13 days after hatching. Previous tests had shown that unfertilized females kept too long in isolation were no longer able to extrude an egg-sac and to produce viable offspring, even though mating occurred. In some cases the females were, however, prevented from mating, since they had extruded very few eggs. A complete egg-sac was never produced without copulation, as it has been observed in other species of *Tisbe*. For *T. clodiensis* from Venice 13 days seemed to be a limit for normal mating to insure a high percentage of offspring. Forty-seven pairs out of 71 were observed mating within the first 20 minutes. Fifty females extruded their egg-sac on the following day, that is, 14 days after hatching. In sample E, females and males had been kept 6 days longer in isolation than the control pairs; although mating occurred 4–5 days later, egg-sac production was delayed only about 2 days

From 51 control pairs 3 females produced an egg-sac in which the eggs failed to hatch and seven females were discarded because of the low number of offspring. The sex-ratio of the progeny produced by 41 control females was calculated to be 47.29% ♀♀ (716 females and 798 males). Also in this case the data are heterogeneous (Table II).

From 71 pairs of sample E the progeny of 56 was considered for a statistical evaluation of the data, since 10 families contained less than 20 individuals. Three females produced an egg-sac, in which the eggs failed to hatch, and 2 females did not extrude an egg-sac. The offspring of the 56 females showed a clear tendency towards an increase of males, that is, 1096 females and 1709 males. As expected in view of the consistent trend in favor of males, a comparison between the sex-ratio of samples E and C yielded a chi-square = 27.273 ($P < 0.001$) (Table II).

TABLE II

Comparison between the mean sex-ratios of the control (C) and experimental (E) series

	No. of families	Total No. adults	% ♀♀	Comparison between sex-ratios
T. dobzhanskii from Anzio				
C	36	1741	41.12	$\chi^2 = 244.092$
E	42	2262	18.61	$P < 0.001$
T. clodiensis from Venice				
C	41	1514	47.29	$\chi^2 = 27.273$
E	56	2805	39.08	$P < 0.001$
T. clodiensis from Ponza				
C	29	1441	40.87	$\chi^2 = 49.751$
E	49	2732	30.01	$P < 0.001$
T. holothuriae from Ponza				
C	29	1493	45.14	$\chi^2 = 25.140$
E	46	2770	37.43	$P < 0.001$

Shifting of the sex-radio towards males was observed in 32.1% families of sample E, whereas in the control series only 14.6% had a significant increase of males (Table I). For these data $\chi^2 = 3.887$ ($P < 0.005$) was obtained. Comparing the average number of males and females per family of samples C and E, respectively, 36.9 and 50.1 individuals, an increase of 13 individuals per family in sample E was observed, largely due to the greater number of males.

T. clodiensis (*from Ponza*)

In the other population of *T. clodiensis*, the average time between hatching of the nauplii and egg-sac production was observed to be 12–13 days. When nearly all females of sample C had become ovigerorus, that is, 13 days after hatching, the isolated males and females were put together, one pair for each dish. Thirty-four pairs out of 60 were observed mating within the first 20 minutes; subsequently fifty-four females extruded an egg-sac on the following day, that is, 14 days after hatching. Thus, males and females of sample E had been kept isolated about 5 days longer than the controls. Although mating occurred about 4 days later, egg-sac production was delayed for only two days, as it had been observed for *T. clodiensis* from Venice.

From 33 control pairs, 2 produced an egg-sac which did not develop, one female extruded no egg-sac, and one gave very few offspring. The sex-ratio of the remaining 29 families was calculated to be 40.87% ♀♀, that is, 589 females and 852 males (Table II).

From the 54 females of sample E the progeny of 49 could be utilized for statistical analysis, since one female did not extrude egg-sacs, 2 gave a reduced number of offspring and 2 others produced egg-sacs, the eggs of which failed to hatch. From 49 families, 820 females and 1912 males were obtained, corresponding to a sex-ratio of 30.01% ♀♀. Differences between the number of males and females of the control and experimental series yielded a highly significant chi-square value (Table II).

In 46.9% of the families of sample E the sex-ratio was shifted in favor of males, while in sample C only 20.6% showed an increase of males (Table I). A comparison of the precentages gave $\chi^2 = 5.379$ and $P < 0.02$. The average number of individuals per family was somewhat higher in sample E (6 individuals), but less than in the case of *T. clodiensis* from Venice.

In order to detect what happens if fertilization is delayed still more, 18 virgin females were kept 14 days in isolation. Immediately after introduction of the males, copulation occurred. Two females did not produce an egg-sac, one female extruded eggs which failed to hatch and one gave very few offspring. The progenies of the remaining 14 families suggested a still greater tendency to produce males than in the experiment described above, the sex-ratio being 27.18% ♀♀. But there was a slight drop in the number of individuals per family. Since the results are based on a relatively small sample a statistical evaluation of the data was omitted.

T. holothuriae (*from Ponza*)

For *T. holothuriae* from Ponza the average time between hatching of the nauplii and egg-sac production was found to be 9–10 days. When nearly all females of the controls had become ovigerous, that is, 10 days after hatching, males and females until then isolated were put together. A period of 10 days seems to be the extreme limit to guarantee normal fertilization, since of the 58 isolated females 8 had produced a small egg-sac in absence of a male 10 days after hatching, thus excluding the possibility that fertilization could still occur. After the males had been introduced only 12 pairs were found mating within the first 20 minutes. This may be due to the fact that mating time in *T. holothuriae* is short and thus, difficult to be seen. Moreover, this species is sexually less active than *T. clodiensis*. From the 50 females, 42 produced an egg-sac one day after introduction of the male, that is, 11 days after hatching. Thus, in the case of *T. holothuriae* the time differences between sample C and E for the moment of copulation and especially for the period of egg-sac production were remarkably reduced, being 4 and 1–2 days, respectively.

From 32 control pairs one female produced an egg-sac which gave no offspring, and two families were discarded. The sex-ratio of the 29 families was calculated to be 45.14% females, based on a total number of 1493 individuals. The data show, however, a strong heterogeneity, 37.9% of the progeny being shifted in favor of females, 24.1% in favor of males, and 37.9% being statistically insignificant (Table I).

From the 50 females of sample E, two extruded no egg-sac, and 2 gave less than 15 female offspring. The other 46 females produced 2770 individuals yielding a sex-ratio of 37.43% ♀♀. Comparing this value with the sex-ratio of the control series a highly significant value of χ^2 (Table II) was obtained.

In spite of the suggestive trend towards males in sample E, the comparison between the percentage of arrhenogenous families of sample C and E, 43.4% ♀♀ and 24.1% ♀♀, respectively, does not result in a statistically significant χ^2 value, since $P < 0.10$ for $\chi^2 = 2.959$.

Also in *T. holothuriae* an increase of 9 individuals per family was recorded in sample E, entirely due to an increase in the number of males.

DISCUSSION

Most of the present knowledge about the effects of a delayed fertilization is based on very few studies carried out with amphibians (Hertwig, 1921; Kuschakewitsch, 1923; Witschi, 1923), butterflies (Seiler, 1920).

Experiments with *Rana esculenta* indicated that overripeness of eggs produces complete reversal of females into males. From normal eggs 53% males were obtained, from overripe eggs fertilized 89 hours later than the controls 100% males hatched (Kuschakewitsch, 1923). Witschi (1924) attributed this sex-reversal to cytoplasmic alterations that affect the medulla more than the cortex in the later part of the development.

Also in the case of the butterfly *Talaeporia* overripeness of eggs produces a majority of homozygous male zygotes, since it favors the permanence of the X-chromosome in the egg. Low temperature (3–5° C) has the same effect (Seiler, 1920).

Shull and Ladoff (1916) who treated the rotifer *Hydatina* with various substances (potassium sulfate, iron chloride, ammonium chloride, dilute bouillon) suggested that the speed of metabolic reactions is responsible for the varying degrees of male production. They assumed that male production is reduced, when the physiological processes are retarded, which they think to be the case for most agents which affect the life cycle.

Similar considerations were made by Takeda (1950) and Egami (1951) who studied the effect of external agents on the copepod *Tigriopus japonicus.* At the end of a long series of experiments they concluded that all suppression of metabolic activity tends to induce feminization. This would correspond to the situation dedescribed for *Hydatina,* but certainly not to that in *Tisbe* and *Talaeporia,* since the delay of fertilization or a low temperature may rather induce retardation of developmental processes. As the above experiments evidenced, the genus *Tisbe*—at least the species taken in consideration—shows a more or less pronounced tendency towards masculinization in consequence of overmaturation of the eggs depending on the duration of the delay.

In fact, in *T. dobzhanskii* where the differences concerning the moment of fertilization and egg-sac production between sample C and E were greater than in the other species, the shifting towards males was statistically highly significant. This is less evident in *T. holothuriae*. While in *T. dobzhanskii* the pairs of sample E had been kept isolated until nearly each female of the control series had given offspring, in *T. holothuriae* the males had to be put together with the isolated females when only half of the control females had produced an egg-sac, since the former began to extrude their eggs without being fertilized. A point which I should like to mention briefly in this context is the choice of two different populations of *T. clodiensis* for the experiment. Former experiments have shown that there is nearly complete reproductive isolation between some geographical populations of this species, and the fact that they differ in their life cycle parameters and polymorphism suggests that they have evolved independently. The present data produce evidence that in addition to the different sex-ratio the population of Ponza appears to be more affected by a delayed fertilization.

With too long a delay the females became unable to produce an egg-sac or extruded only very few eggs which failed to hatch (*T. dobzhanskii* and *T. clodiensis*), or a small egg-sac was extruded before mating occurred (*T. holothuriae*).

The shifting of the sex-ratio in consequence of overmaturation is accompanied in *Tisbe* by another phenomenon, namely, increase of the number of offspring per family, largely due to the greater number of males. Thus, the number of females per family remained nearly equal in the control and the experimental series (*T. clodiensis* and *T. holothuriae*). This could be due to a reduced mortality in sample E, as stated for *Rana esculenta*, where 6% mortality was observed in the normal culture and only 4% in the culture from overripe eggs (Kuschakewitsch, 1923). It is however, somewhat difficult and rather laborious to solve this question in *Tisbe*, since the eggs of an egg-sac cannot be counted without destroying the egg-sac. On the other hand, comparing the sex-ratio data, as far as it is possible, with those obtained from other experiments, it can be proved easily that the number of individuals per family in the control series was rather high or at least normal, so that differential mortality may probably be excluded.

It seems, although there is no statistical proof, that more eggs are produced when the fertilization is artificially delayed. Since the males and females of sample C and E were carefully observed when they extruded their egg-sacs, it was noticed that the egg-sacs of sample E were bigger. This would suggest that there is a continuous production of eggs for a time in the absence of fertilization. The oviducts of the isolated females were, in fact, filled with eggs, more so than in the case of normally fertilized females. The delay of fertilization at the beginning of the reproductive cycle seems to affect also the production of the second egg-sac. Fifteen females of *T. holothuriae* and 17 females of *T. clodiensis* from Venice (sample E) were kept until they had produced a second egg-sac. The results, which are too few to be statistically evaluated, indicated that shifting of sex-ratio towards males remains approximately constant and that the number of individuals per family increased to 27 individuals in *T. holothuriae* and to 14 individuals in *T. clodiensis* in comparison with the first egg-sac of sample E. It cannot be decided, however, if the rather strong increase of individuals and the shifting of the sex-ratio towards males is maintained during the whole reproductive cycle. In order to resolve this question 4–8 egg-sacs per female should have been examined.

The physiological process which is at the base of the phenomenon described above cannot be explained by the present data. The findings suggest, however, an adaptive nature for such a mechanism. According to the hypothesis of sex-determination in *Tisbe* where the greater proportion of males is a function of homozygosity, the high degree of females in nature would be a consequence of the high level of heterozygosity permitted by a favorable environment (Battaglia, 1964). Battaglia continues (page 459): "Even if the number of females should greatly exceed that of males, the reproductive rate of the population would not be affected, since in *Tisbe,* each male is able to fertilize several females." This interpretation would not apply to species such as *T. clodiensis* and *T. dobzhanskii,* since a strong decrease of males in a population—in nature or in the laboratory—would delay fertilization of most of the females to a relatively high degree; first, because *T. clodiensis* and *T. dobzhanskii* tend to mate for a relatively long time (sometimes 24 hours and longer), and, secondly, even though a male is able to fertilize several females, not all males have two spermatophores and it takes some time to produce a new spermatophore. Studies with *T. reluctans* and *T. persimilis* proved that a new spermatophore is produced after about 2 days (it is probable that there are differences between the various species). A strongly reduced male population would thus induce a delay in fertilization and as a consequence an increase of males in the following generation which should guarantee a normal fertilization of the females, as long as the delay is not too long. At this point I would like to return to a question mentioned before, *i.e.*, the duration of the influence of overmaturation. Even if subsequent egg-sacs (3rd, 4th, *etc.*) showed an increase of females, their normal fertilization would be assured because of the abundance of males produced in the first two egg-sacs. These considerations so far have to be restricted to *T. dobzhanskii, T. clodiensis,* and *T. holothuriae.*

Another important factor in this context is certainly provided by population density. Where densities are low, the chance of females being normally fertilized are reduced. According to Battaglia (1964) inbreeding following the low population density would increase the proportion of males and thus, give a higher proportion of fertilized females.

This may be the case for species like *T. reticulata* and *T. gracilis* where inbreeding increases the proportion of males, but not for *T. clodiensis* where inbreeding has no effect on masculinization (Fava, personal communication). In this species the mechanisms of "overmaturation of eggs" may operate as a consequence of a reduced population density. It would be helpful to know if *T. dobzhanskii* is affected by inbreeding and on the other hand, if *T. reticulata* and *T. gracilis* are influenced by a delayed fertilization, in order to understand to what degree sex in *Tisbe* may be genetically and environmentally determined.

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SUMMARY

Delay in fertilization of the females of *Tisbe dobzhanskii* from Anzio, *T. holothuriae* from Ponza, and *T. clodiensis* from Venice and Ponza was found to affect the sex-ratio of these populations.

The degree of the influence depended largely upon the lateness of fertilization and, thus, upon the biology of the species.

Especially in *T. dobzhanskii* shifting of the sex-ratio in favor of males was highly significant. In the other populations it was less evident, but still significant. If the delay of fertilization was too great, the females were no longer able to produce viable offspring.

In addition to the shifting of the sex-ratio towards males an increase of offspring per family was observed.

The possible adaptive nature of the phenomenon is discussed.

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2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

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